

A health warning on bioterrorism

Scientists should be careful not to exaggerate the usefulness of biological agents as terrorist weapons, and focus their efforts on developing an effective international verification regime.

The spectre of bioterrorism, in which toxic biological agents are released into the atmosphere in an attempt to injure or kill civilians, is not one that tends to elicit a measured response. But a British Royal Society report issued last week manages to sidestep the hysteria that can overtake discussion of the topic. In warning about the dangers of unnecessary panic, suggesting sensible precautions by public-health agencies, and emphasizing the central importance of current negotiations to allow the verification of the Biological Weapons Convention, the Royal Society has managed to hold this volatile issue in badly needed perspective.

The society's report, *Measures for Controlling the Threat from Biological Weapons*, points out that "the effectiveness of [biological weapons] spread in aerosol form against human populations in war or by terrorist activity has not been proven". While rightly warning against complacency about biological weapons, the report adds that "it would seem prudent not to overestimate them".

It could have added that the perceived potential of biological weapons as a terrorist threat seems to grow in proportion to one's distance from actual terrorist activity. The United States has recently made its response to bioterrorism a significant national priority, with an annual budget of \$1.4 billion — about a quarter of it going to research. Yet biological weapons have rarely been used by terrorist groups. The isolated attempt by a well-financed cult to use sarin nerve gas against commuters in Tokyo in 1995, after many previous tries, only emphasizes their relative lack of political or military utility.

The committed terrorist tends to be a ruthless pragmatist with a

clear political agenda. Explosions and even airline hijacks can meet that agenda by attracting publicity and dramatizing specific demands. Letting off poison gas in a public place is a much less effective way of doing either. Even in the movies, terrorists are seldom motivated by the indiscriminate targeting of civilian populations for its own sake.

No such reservations, however, were acknowledged in a report last year from the US Institute of Medicine, *Chemical and Biological Terrorism*. This culminated in an impressive laundry-list of research needs presented by such 'threats'. Tens of millions of dollars of extra research funding have subsequently been ploughed into this topic, and Washington conferences on the subject have been crowded out by hundreds of consultants, contractors and scientists keen to join the action. The creation of demand for such programmes, and their subsequent exploitation, has not been an edifying sight.

The best thing that can be done to safeguard the public from biological weapons, as the Royal Society correctly notes, is for the signatories to the Biological Weapons Convention to agree on a verification regime for the treaty, as they will try to do at a meeting in Geneva next week. Sensible precautions should certainly be taken by military and public-health agencies to prepare for certain types of biological attack. But the panic of an ill-informed public, as the society suggests, could be at least as dangerous as an attack itself — for example, by the disruption caused by mass evacuation in response to a perceived threat. That places an onus on the scientific community to keep such threats in perspective. ■

Genome sequencing for all

The successful sequencing of a plant pathogen by Brazilian researchers is a political as well as a scientific achievement.

There is a common misconception that only advanced industrialized nations have the wherewithal and skilled human resources needed to achieve cutting-edge science. This misconception is fanned by the number of researchers from developing countries who find it necessary to obtain their research training abroad — and frequently decide not to return, citing a lack of scientific opportunity. But it is given the lie by a paper published in this issue which describes the result of a project carried out by a consortium of research centres in the state of São Paulo in Brazil to sequence the bacterium *Xylella fastidiosa*. This bacterium causes a disease that affects citrus fruit and other important crops, resulting in many millions of dollars of damage each year (see page 151).

As the first public sequence of a free-living plant pathogen, the paper represents a significant scientific milestone. But it also sends a clear political signal, namely both the desire and ability of countries such as Brazil to play in the big league. The sequencing project was deliberately chosen by the project's main funding agency, FAPESP, to play a catalytic role in helping research teams equip themselves for the challenge of the post-genome era. It was also intended to send a signal to Brazil's young scientists that they do not need to leave the country to engage in world-class science. In both respects, it appears to have succeeded.

Of course, sequencing the genome of the bacterium is only the first step towards controlling the damage that it causes. The next is to apply functional genomics to understand how the bacterium's genes operate, opening up routes to possible intervention in limiting its spread by insects. Eventually, knowledge of the genome could provide the information required to breed resistant varieties of the affected crops. This raises a separate set of challenges — to persuade the Brazilian public that transgenic plants can play an important economic role, and at the same time to take firm steps to avoid untoward social and environmental consequences (see page 115).

On the technical side, much of this lies some way in the future. But the success of the *X. fastidiosa* project has already attracted significant expressions of interest for similar projects from other parts of the farming community — one proposal high on the list is for the same sequencing centres to turn their attention to chicken expressed sequence tags (ESTs). It has also given rise to the welcome and relatively unusual phenomenon of an agency in the advanced industrialized world — in this case the US Department of Agriculture, worried about the impact of a variety of *X. fastidiosa* on citrus crops in California — contracting research from a developing country. Both achievements endorse Brazil's determination to enter the post-genome age hand-in-hand with scientists in richer countries. ■





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German agencies sound alarm on risks of broad gene patents...

Quirin Schiermeier, Munich

The heads of two key German science organizations have warned that the award of broad patents on gene sequences could stifle basic genomics research and competition for pharmaceutical innovations.

They are urging Wolf-Michael Catenhusen, the science ministry's secretary of state for research, to explore ways of requiring national and European patent rules to be interpreted in a way that forbids patents covering all possible applications of a particular gene sequence. Instead, patents should be restricted to identified functions.

The warning has come from Detlev Ganten, head of the Max Delbrück Centre for Molecular Medicine in Berlin and president of the Helmholtz Association of National Research Centres, and Ernst-Ludwig Winnacker, president of the Deutsche Forschungsgemeinschaft, Germany's main research funding agency.

Speaking at a press conference last week following the announcement of Germany's new genome research strategy (see *Nature* 406, 6; 2000), they argued that it should no longer be possible to obtain patent protection on all potential functions of a biological material — such as a gene — and that patents should be restricted to specific functions.

According to the European Patent Convention, product patents on the commercial use of substances such as chemical compounds or biological material — including DNA — may be granted only if the inventor can identify a specific function.

But Winnacker and Ganten criticize the fact that European gene patents can cover all of the potential functions of a DNA sequence. As a consequence, they say, the owner of a patent on a gene sequence could block the commercialization of any newly discovered function of this sequence, or demand a licence fee.

Winnacker referred to the patent held by the American company Human Genome Sciences on the chemokine receptor CCR5, whose role as a 'revolving door' for the HIV virus was completely unknown when CCR5 was first described as a cell receptor for lymphocytes (see *Nature* 404, 322; 2000). Such



Protective action: Greenpeace is not alone in its protests over the effects of gene patents.

broadly worded patents could deter scientists from researching genes which, for example, overlap with DNA sequences already protected by a patent.

The issue will be discussed over the coming months by patent experts from the federal ministries of science, health and legal affairs as they prepare to adopt as national law the European Union's (EU's) directive on the legal protection of biotechnological inventions, passed in 1998.

The directive will harmonize biotechnology patents in the EU. In principle, patent

officers determine whether the breadth of a patent claim is too broad, using the conventional criteria for judging patent applications. But national governments can still require an interpretation of European-level rules that reflects experience of new and rapidly growing areas such as biotechnology.

Jens Reich, a bioinformaticist at the Max Delbrück Centre, says he would welcome any move to limit the scope of patents on gene sequences. He says that a growing number of "equally vague and broad" claims slipping through the patent offices' examination formalities could block real innovation in genomics. "In an ideal world," says Reich, "a patent-relevant description of a gene function should require a careful cell-biological examination of the physiological effects."

But the German pharmaceutical industry wants to see the EU directive adopted as it stands, and does not want specific restrictions added to biotechnology patents. Dieter Laudien, head of the patent committee of the association of research-based pharmaceutical companies in Germany, says that a special treatment of biotechnology patents would violate the international agreement on trade-related aspects of intellectual property rights (TRIPs), which says that there must be no substantial differences between patent rules in different technical fields. ■

...as spending goes up for research

Molecular medicine and biotechnology are the big winners in Germany's proposed federal science budget for 2001. As long as it is approved by parliament, total federal spending on science will rise next year by 5.3%, to DM15.37 billion (US\$7.5 billion).

The increase means that the coalition government of Social Democrats and Greens remains loyal to its 1998 election promise to significantly increase spending on research. Within this total, money available for university

building and large equipment — hitherto notoriously underfunded items — is to increase by DM215 million, and will next year total DM2.22 billion.

The new budget also illustrates Germany's efforts to become a world leader in genomics research (see also *Nature* 406, 6; 2000). Since 1998, project money available for biotechnology and molecular medicine has risen by 30.4% and 47.8%, respectively, now reaching DM220 and DM96 million.

Institutional funding of the main research organizations will also rise. The Deutsche Forschungsgemeinschaft, the main funding agency for university research, will get DM1.23 billion, 5% more than last year. The Max Planck Society, Germany's largest non-university basic research organization, will receive a more modest increase of 3.9%, taking its funding to DM888 million. Both organizations will receive similar sums from Länder (state) budgets. **Q.S.**

Internet publishing camps renew hostilities

Colin Macilwain, New York

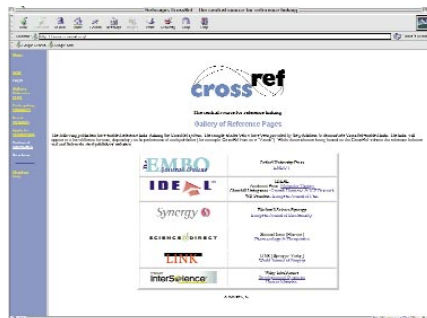
The simmering tension between commercial publishers and those who believe that scientific literature should be available free on the Internet boiled over last week — at a meeting held to promote dialogue between the two groups.

Disagreement was fuelled by the news that six months after the US National Institutes of Health (NIH) launched PubMed Central (PMC) — a forum for the open posting on the Internet of research results already published elsewhere — few scientists are so far using the site.

At a meeting held at the New York Academy of Medicine, Pieter Bolman, president of Academic Press, one of the leading commercial publishers, said that PMC had been “asking for trouble” by trying to change simultaneously both the dominant distribution and business models for the communication of scientific information.

Bolman said that CrossRef, an information-exchange network agreed by publishers last November (see *Nature* 402, 226; 1999), contained a thousand times as much published material as PMC. He suggested that the latter, having “served its purpose” by helping to catalyse CrossRef (of which *Nature* is a member), should abandon its wider ambitions and join the publishers’ network.

But advocates of PubMed Central say the site still has time to develop an audience.



In front? The publishers' CrossRef claims to be outstripping the NIH's PubMed Central.

They express confidence that the participation of new publishing projects committed to open access — such as BioMedNet Central, part of the UK-based Current Science Group, which organized the meeting — will soon bring it new momentum.

Pat Brown, a geneticist at Stanford University and early advocate of PubMed Central, called on scientists to “take full control of the publishing process” and “insist that it is free, untaxed by the parasites in the publishing world”.

Harold Varmus, president of the Memorial Sloan-Kettering Cancer Center in New York and former director of the NIH, said that PubMed Central had met with more resistance than he had anticipated. He told the meeting that the project's basic vision

was unchanged, but that “what we've retreated to, or I should say progressed to, is a short-term view” of the project as “a public vehicle, with government finance, to help distribute information that is in existing journals”.

Varmus says that researchers want to browse top journals — which he expects will survive — but to search systematically the material that currently appears in a vast array of middle-ranking journals. “BioMed Central is going to have a devastating impact on the mid-ranking journals,” he says.

Faced with the prediction that many scientific societies' journals will not survive the resulting fall in individual subscriptions, some speakers defended the editorial function of such journals.

George Lundberg, for example, former editor of the *Journal of the American Medical Association* and editor-in-chief of Medscape, an Internet-based medical database, said that the editor's role, “which much of [this] discussion is intended to abolish, sits at the middle” of scientific publishing.

And Marc Brodsky, executive director of the American Institute of Physics, predicted that BioMed Central, with its plans for ‘open’ peer review and its inclination to publish most of what it receives, would end up publishing material akin to conference proceedings — which, he says, are “not looked on very well” by the scientific community. ■

► <http://pubmedcentral.nih.gov>

US scientists seek more funds for high-tech equipment

Paul Smaglik, Washington

The largest professional body representing US researchers in the life sciences is urging the National Institutes of Health (NIH) to nearly quadruple its contribution to a scheme that helps investigators pay for equipment costing over \$100,000.

A survey carried out by the Federation of American Societies for Experimental Biology (FASEB) found that about three-quarters of the 508 NIH-supported scientists who replied felt that the scheme, known as the Shared Instrumentation Grants (SIG) programme, is inadequately funded and managed.

Almost half of the respondents to the survey, the results of which were released in Washington this week, said a lack of financial support meant that their laboratories were not able to add research technologies as fast as they would like.

Although the NIH's overall budget has risen by 15 per cent in each of the last two years, FASEB points out that money for equipment has not kept pace. In particular,

the SIG programme, which allows researchers already supported by NIH grants to split the cost of equipment costing over \$100,000, has fallen in real terms over the past decade.

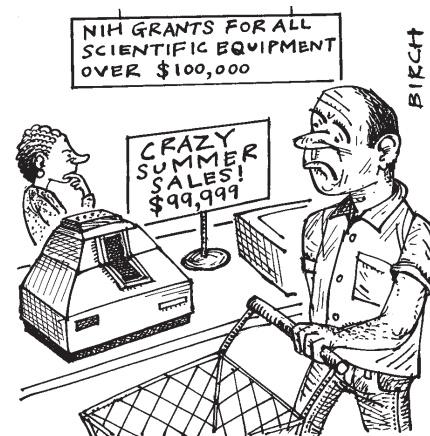
SIG was funded at \$32.5 million for the financial year 1990 and \$43.1 million in 2000; it suffered during the budget restrictions of the early 1990s and is only now beginning to recover. FASEB recommends that the programme receive \$150 million in the financial year 2001.

Researchers are concerned about their ability to pay for the high-tech — and expensive — techniques on which they increasingly depend. In the past, such sentiments have been mostly anecdotal, so FASEB launched its survey last autumn to unearth more concrete evidence. The survey found that 84% of the respondents said that shared equipment was “extremely important” for their research.

David Speicher, the study's lead author, calls the \$150 million demand “pretty conservative”, as it is based only on the

number of investigators receiving R01 grants from the NIH.

“The scientific endeavour is moving in a direction where modern, expensive equipment is essential,” says Speicher, a professor of structural biology at the Wistar Institute in Philadelphia. “If you don't have the equipment, it impairs the research.” ■



Mbeki gives AIDS scientists the cold shoulder

Michael Cherry, Durban

South African President Thabo Mbeki has answered critics of his stance on the relationship between HIV and AIDS by ignoring them. Opening the 13th international AIDS conference in Durban last Sunday, he made no reference to an appeal from scientists to revise his position (see *Nature* 406, 15–16; 2000).

Mbeki has generated widespread concern by refusing to endorse the view that HIV causes AIDS. His decision to stick to this position — although he did refer to “HIV/AIDS” in his speech — disappointed many delegates. They were also frustrated by the lack of any mention of how Mbeki’s government hopes to reduce the transmission of HIV from pregnant women to their babies.

“I’m disappointed that an opportunity was lost, both to set the record straight on the causation of AIDS, and to present a concrete plan to reduce mother-to-child transmission,” says Slim Karim, head of the HIV/AIDS unit at the South African Medical Research Council (MRC).

“Mbeki waffles on, while Rome burns,” says paediatrician Glenda Gray, director of the perinatal HIV/AIDS unit at Chris Hani Baragwanath Hospital in Johannesburg.

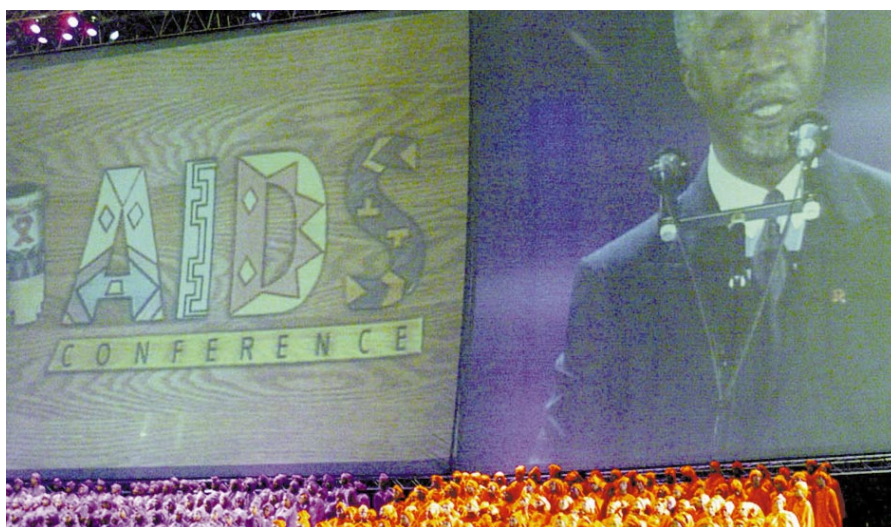
Mbeki focused on the well-documented impact of poverty on AIDS. “What he said is accurate; it’s just what he didn’t say that is the issue,” says Lynne Mofenson of the National Institute of Child Health and Development at the US National Institutes of Health. But she adds a hope that, “as he gains more insight, he will recognize that perinatal transmission is something that can be prevented, and hopefully act accordingly”.

Mbeki said his advisory panel on AIDS, which met for the second time last week (see *Nature* 406, 3; 2000), had agreed to report back by the end of the year on “the reliability of and the information communicated by our current HIV tests and the improvement of our disease surveillance system”.

The panel has been sharply split between those who accept the HIV hypothesis and the ‘dissidents’, led by Peter Duesberg of the University of California, who challenge it.

The meeting hammered out a broad proposal to test the accuracy of HIV testing in South Africa by comparing the results of ELISA (enzyme-linked immunosorbent assay) tests for HIV with those of other HIV tests, including the isolation of the virus.

MRC president Malegapuru Makgoba presented evidence to the panel that the percentages of false positives — the concern of the dissidents — and false negatives recorded in South African laboratories were similar to those in Western countries. Despite this, the panel agreed to test the accuracy of ELISA “in the interests of keeping the peace”, according



Don't turn away: President Mbeki addressed the Durban conference on its opening day.

to Karim, one of its members.

Khotso Mokhele, president of the National Research Foundation and head of the panel’s secretariat, said after the meeting that he hoped the testing of screening procedures might persuade one side to “shut up once and for all”. But observers say dissidents and orthodox members are unlikely to agree on what isolation of the virus actually means.

South African HIV statistics are based on antenatal surveys analysed at laboratories across the country, using a standard protocol. Researchers would like to see testing centralized, eliminating the marginally differing levels of false negatives and positives recorded. Although panel members agree this is desirable, it would be unlikely to significantly alter estimates of HIV prevalence.

Mbeki referred in his conference speech to the need for accurate statistics rather than “estimates based on projections” — another of the dissidents’ arguments — but ignored statistics on changes in mortality obtained from data compiled by his own Department

of Home Affairs.

These show that deaths in South Africa among men in the 15–49 age group have increased during the past decade from 48% to 87%, while those for women in this age group have risen from 27% to 68%.

Although Mbeki did not mention the issue, mother-to-child transmission of HIV seemed set to dominate the conference. Several South African studies were presented which showed that the antiretroviral drugs AZT and nevirapine are roughly similarly effective in preventing mother-to-child transmission, even though nevirapine is only a fraction of the cost of AZT.

One outstanding issue being used by the South African government to justify not supplying either drug to pregnant women is the possibility of subsequent transmission via breastfeeding. But new evidence being presented at the conference suggests that this can be significantly reduced by early weaning, where formula feeding is possible. ■

♦ <http://www.aids2000.com>

UK studentships get funding boost

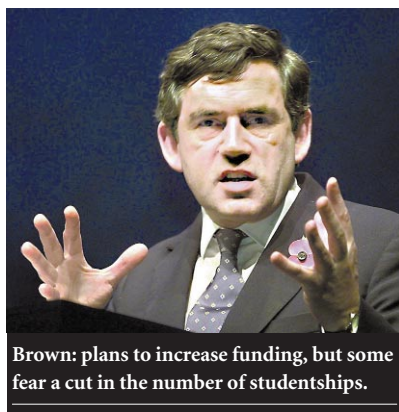
Natasha Loder, London

The basic annual award of British research students is set to rise by 23 per cent in real terms over the next three years. The deal means that over five years the minimum government research studentship will have risen from £5,500 (US\$8,300) to £9,000 a year.

The UK Treasury announced the news last week together with a two-year, £1 billion package of investment in buildings, laboratories and equipment. The deal includes £230 million from the Wellcome Trust.

In recent years, both industry and academia have expressed concern over the quality of research students. Extra money has helped: in 1998, following an increase of £1,000, the number of unfilled studentships at the Engineering and Physical Sciences Research Council halved (see *Nature* 401, 519; 1999).

But a study commissioned last year by the Office of Science and Technology showed that undergraduate debt had a significant impact on the supply of research students, and revealed a shortage of PhD candidates



Brown: plans to increase funding, but some fear a cut in the number of studentships.

► in many areas, including the biological and physical sciences.

The government has not yet said how much extra money will be available to cover the increases, leading some to fear that student numbers could fall. Earlier this year the life-science community called for a pay increase, even if it meant cutting back on numbers (see *Nature* 403, 347; 2000). But the Treasury has denied that this is their intention. A spokesperson said the aim was to have more and better research students, and that "this does not mean fewer studentships".

Bob Price, the head of human and corporate resources at the Biotechnology and Biological Sciences Research Council (BBSRC), welcomed the announcement with "open arms" and expects it to raise the quality and quantity of research students.

But earlier this year John Taylor, the director-general of the research councils, decided that stipends did not have to be harmonized, and that individual councils could choose whatever level they thought appropriate. Accordingly, the BBSRC's minimum went up to £7,380 and is under review. Price says it could increase beyond the amount announced by the Treasury, but that this would "need a reduction in the number of places".

But although welcoming the money in principle, many scientists are reserving judgement until the small print is revealed later this month — particularly as the announcements appeared as a leak to two newspapers of a speech by Gordon Brown, the Chancellor of the Exchequer, and as selective details released by the Treasury.

Indeed, science may not be a net beneficiary. Money could be redistributed from other budgets, as was the case with government departmental spending (see *Nature* 404, 909; 2000), or clawed back elsewhere. The full results will be known when the government releases details of the Comprehensive Spending Review in a few weeks.

Tangled tale of a lost, stolen and disputed coelacanth

Heather McCabe, Paris & Janet Wright, London

Strange stories have long circulated around the coelacanth, the 'living fossil' fish discovered off the coast of South Africa in the 1930s.

The latest are claims by French researchers who say they were the first scientists to find the Indonesian variety, in 1995. But the photograph they sent to *Nature* to support their claims has been denounced by another researcher as a fake.

Until recently, coelacanths had only been found on the west side of the Indian Ocean. The first recorded Indonesian sample was discovered by a US biologist, Mark Erdmann of the University of California at Berkeley, who published news of his find in *Nature* (see *Nature* 395, 335; 1998).

In a recent submission to *Nature*, the French team — Bernard Séret, Laurent Pouyaud and Georges Serre — say they were unable to register their specimen in 1995 because it failed to reach the museum to which it had been sent. They say Serre photographed the fish at the time, then lost the picture (their only other evidence) while moving house, and only found it again this year.

But *Nature* staff noticed that the fish in the new photograph appears virtually identical to the one caught by Erdmann. When contacted, Roy Caldwell, a co-author of the 1998 paper, scrutinized the photograph via a picture-editing computer program and said, "I am 100% certain the image is a fake".

When Erdmann spotted a coelacanth off the northeast coast of Sulawesi in 1998, his find expanded the geographical distribution of the fish by roughly 10,000 km. But Georges Serre, a consultant for what is now the French Institut de Recherche pour le Développe-

ment (IRD), has long claimed that a 10-kg specimen was caught in 1995 in the Bay of Pangandaran, in Southwest Java.

Serre says he gave the specimen to a fisherman to hand over to the Indonesian fishery service. But the man gave it to a museum, from which it was stolen. Only recently, according to Serre, did Pouyaud, an IRD geneticist in Jakarta, track down the coelacanth to a private collection, whose owner refuses access to the fish.

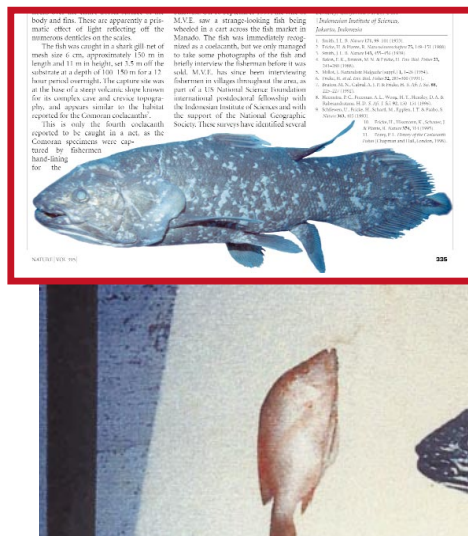
The French team's finding, if confirmed, would further extend the distribution of this elusive creature, as it was caught more than 2,000 km from the spot where Erdmann found his 1998 specimen, suggesting a large distribution in the Indo-West Pacific region.

The whole issue is already shrouded in controversy. After analysing the specimen that Erdmann had given to the Indonesian authorities, Pouyaud and his colleagues at the Indonesian Institute of Science named it as a distinct species, *Latimeria menadoensis* — to the chagrin of Erdmann, who had been analysing tissue samples independently.

Caldwell says that coelacanths have highly individual spot patterns; the pattern in the two photographs is virtually identical. The bulky fish in the 1998 photo was swimming in the sea, yet is seen in the identical position, in the new photo, while lying on a slab. Shadows and other details seen when the image is magnified add to Caldwell's impression that the new photograph has been manipulated.

Serre still claims that the photograph is authentic, though he now says it was taken by a friend who later died and whose widow gave it to Serre before moving abroad.

Séret, who is an ichthyologist at the Muséum National d'Histoire Naturelle de Paris, admits that the two photographs do appear to show the same fish. "This is very embarrassing," he says.



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Vive la différence: The French fish (right) bears a striking resemblance to one that appeared in *Nature*.

Sweden streamlines research administration

Declan Butler

A decade of uncertainty over who should control Swedish science could be about to end. The Swedish parliament is considering a bill to replace the country's myriad research councils with just four.

A single agency, the National Science Council, would support basic research. One applied agency would cater for social and working-life issues, another for environment and agriculture, and a fourth for industrial research.

Research has become a political hot potato in Sweden. A lack of economic growth during the early 1990s was accompanied by a 30% reduction in research spending. The centre-right coalition decided in 1994 to use US\$800 million, raised through a tax on industry, to fund a Foundation for Strategic Research. This resulted in a controversial shift towards greater support for research with socioeconomic goals.

In 1998, a parliamentary report called for a re-emphasis on basic science, and a radical streamlining of the various agencies within just four councils under the ministry of education, covering applied and basic research.

A new ally came with the return of the Social Democrats in late 1998, when 35-year-old Thomas Östros became minister for education and science. Östros has promised an increase of almost 10% in the basic research budget by 2002. Sweden's total spending on research, at \$59.48 billion or 3.6% of gross domestic product, is already high by European standards.

The new bill, endorsed by the Green and Left parties, and presented to parliament by Östros in June, adopts many of the conclusions of the 1998 report.

Being a small country, Sweden needs a single highly competitive system for distributing research funds across the universities, says Dan Brandström, chairman of the committee that will implement the new structure if the bill is passed as expected. "Before, we had a very fragmented system."

The National Science Council will replace the current Council for Planning and Coordination of Research, the Council for Research in the Humanities and Social Sciences, the Medical Research Council, the Natural Sciences Research Council and the Research Council of Engineering Sciences. In line with the 1998 report, scientists should be in charge: most of the governing board will be elected by the research community.

Lars Nilsson heads the 54-year-old Swedish Natural Science Research Council, which will disappear under the new structure. He says that it is too soon to predict the outcome of the proposed change, but it has the "potential to be something much better".

The streamlined structure should allow



Östros: accepted plans for a single agency.

coordinated use of funding, says Nilsson, and makes sense in an era where research proposals often span sectors that are currently covered by separate organizations.

"The change is positive," says Torvard C. Laurent, professor emeritus at the University of Uppsala. "The new organization will to a large extent be run by scientists elected by the academic community," he adds, noting that the former government had politicized the research agencies.

"The cooperation between the ministry and science is more relaxed now," Nilsson agrees. He believes that the improved state of the economy has also given the present government greater room for manoeuvre.

The former research councils will survive as subunits of the council covering humanities and social sciences, medicine, and 'natural and engineering sciences'. The difficulty will be in achieving a new organizational culture, says Brandström. "The new agencies need to be more proactive. Until now they have been reactive to applications coming from funding proposals."

The agencies will be run by the ministry of education and science. But the ministry of industry will oversee the new national agency for research and development.

"It is a compromise," says Brandström. "The ministry of education and science has one main body, industry the other."

Scientists back GM for Third World

David Dickson, London

An independent panel of leading scientists from both industrialized and developing nations this week endorsed the use of genetically modified (GM) crops to meet the food needs of the world's poor.

But the panel, set up by seven national academies of science, also urged private industry to share GM technology with "responsible scientists" to alleviate hunger in developing nations.

The panel, intended to offer "scientific perspectives" on the role of GM technology in world agriculture, was set up last year by Britain's Royal Society, the national academies of the United States, Brazil, China, India and Mexico, and the Third World Academy of Sciences.

"We felt that this was a good opportunity to provide a technical reflection on the GM debate," says panel member José Fernando Perez, scientific director of the Brazilian research agency FAPESP. "In Brazil, the issue has been almost entirely discussed in political terms; we have never had a technical debate."

The report argues that, among other goals, GM technology should be used to reduce the environmental impact of agriculture. It also calls on countries to introduce systems to monitor the potential human health effects of transgenic plants.

On the question of patents, it says that unless certain plant-modification technologies are extensively licensed or provided without payment to the developing world, "they are unlikely to



Seeds of change? GM crops might benefit farmers such as this Brazilian rice-grower.

benefit the less developed nations of the world for a long time".

Critics complain that the report legitimizes high-tech solutions such as GM without giving due weight to more locally appropriate solutions. "Even if the science is sound, this cannot stand alone without questioning the power that lies behind the market approach to global hunger," says Mark Curtis, of the international development agency ActionAid.

But panel members say the report takes care to place the technical dimension of GM crops in a broader context. It demands, for example, "special exemptions" for poor farmers to protect them from "inappropriate restrictions on propagating their crops".

German council urges collaboration to boost science output

Munich The Wissenschaftsrat, Germany's influential science council, has recommended that the successful cooperation between universities and non-university research institutes on German work on the Human Genome Project should be used as a model for other commercially relevant areas of research, such as semiconductors and medical technology.

The recommendation is included in long-awaited proposals published this week on the future development of Germany's science system. The president of the Wissenschaftsrat, historian Winfried Schulze, says Germany must overcome the problems caused by the fragmentation of its research efforts that result from the independence of its universities and the existence of several science funding agencies, each with its separate budget.

Scientific output could be optimized by stimulating more collaboration and competition between different parts of the science system, including the private sector, according to the report. The Wissenschaftsrat also suggests limiting the lifespan of new institutes to around 15 years,

with any extension requiring scientists to justify their research in terms of its relevance to society.

Security stand-down called at another US national lab

Washington Classified research was suspended at the Pacific Northwestern National Laboratory in Richland, Washington, after lab managers discovered that a photocopy of a classified document had been inadvertently left in an unclassified section of the laboratory.

According to a spokesman for the laboratory, the security stand-down was ordered by laboratory director Lura Powell on 3 July, after a member of staff reported the location of the document. Classified research, which accounts for 13% of the laboratory's budget, will resume this week after staff have been briefed on security procedures.

Indian genome research plans grow

New Delhi Indian scientists are asking the government to launch a project to sequence the genome of *Plasmodium vivax*, the parasite responsible for 80% of malaria cases in India. "The project is do-able," says a spokesperson for the Indian Institute of Science in Bangalore. "We have three laboratories where

it can be done, at a modest cost of \$4 million."

Meanwhile, Nicholas Piramal, a company based in Mumbai, is to invest \$25 million over nine years in developing genetic medicines. In particular, results from the Human Genome Project will be used to investigate the genetic basis of the traditional Ayurvedic system of medicine. "Ayurvedic practitioners never give the same drugs to all patients; they are tailor-made to suit individuals," says a company spokesperson. "Our research will find out the genetic basis of these drugs."

US human protection office halts clinical trial

Washington A US federal agency has suspended 75 clinical trials at the University of Oklahoma's medical school in Tulsa after allegations that a cancer trial there was endangering patients' safety. Twenty-six melanoma patients died during a three-year trial, which involved injecting them with a trial vaccine.

So far there is no evidence that the vaccine contributed to the deaths. The suspension is the first major action by the Office for Human Research Protection. The Department of Health and Human Services set up the office to give stronger supervision of clinical trials, following the first death directly attributed to gene therapy last autumn.



Pi-eyed: Crown Prince Willem Alexander unveils the new tombstone for mathematician van Ceulen (inset).

Mathematical epitaph rises from the dead

London The German–Dutch mathematician Ludolph van Ceulen (1540–1610), who calculated π to 35 decimal places, was last week given a new gravestone in St Peter's church, Leiden, by the Dutch Crown Prince Willem Alexander (see above). Van Ceulen made his calculation using a polygon of 2^{62} sides. The results were engraved on his original tombstone, which was lost. Recently, a chance discovery of the epitaph's text in a book by an eighteenth-century English traveller allowed the stone to be reconstructed.

Royal Society urges calm over biological weapons

London Scientists and government should not exaggerate the effects of biological weapons — although complacency would be irresponsible — according to Britain's Royal Society. The conclusion comes in a report issued following a meeting last year with the US National Academy of Sciences and France's Académie des Sciences.

The group — chaired by Harry Smith, emeritus professor of microbiology at the University of Birmingham — says that genetically modified agents are some years away, and that weapons that would only affect specific ethnic groups are “in the realms of fiction”. It calls for a “scientifically sound and realistic assessment” of the effects of the weapons most likely to be used, and for strong efforts to ensure ratification of the Biological Weapons Convention verification protocol (see *Nature* 391, 831; 1998).

Fingers crossed for satellite launch

London European scientists are nervously awaiting the launch of the first pair of a set of space weather satellites, known as Cluster II, this Saturday in Kazakhstan. The first version of the entire mission of four identical satellites

was lost in 1996 when the rocket carrying them exploded (see *Nature* 381, 541; 1996).

Cluster II was rebuilt in only four years, and this time will launch in two pairs, on Russian-made Soyuz rockets that have a reliable track record. The second launch is set for 9 August. If successful, the four spacecraft will send back data on how the Earth's magnetosphere interacts with the solar wind. The launches will be broadcast live on the Internet.

► <http://television.esa.int>

Report calls for laws to govern space research

Paris International legislation is needed to ensure sound science in space, according to a report on the ethics of space exploration. The report is published by the European Space Agency (ESA) and the United Nations Educational, Scientific and Cultural Organization as a preliminary stage to worldwide consultations on the ethics of space research.

The report, presented on Monday in Paris by Alain Pompidou, former member of the European parliament, and Antonio Rodotà, ESA's director-general, also recommends that observation of the Earth's environmental problems should be a major goal of space science, and that poorer nations should have equal access to satellite data.

Chemistry meets computing

If individual molecules can be made to process information, they could be the answer to the computer industry's prayers. Philip Ball examines the field of molecular logic, which is at last recording some significant achievements.

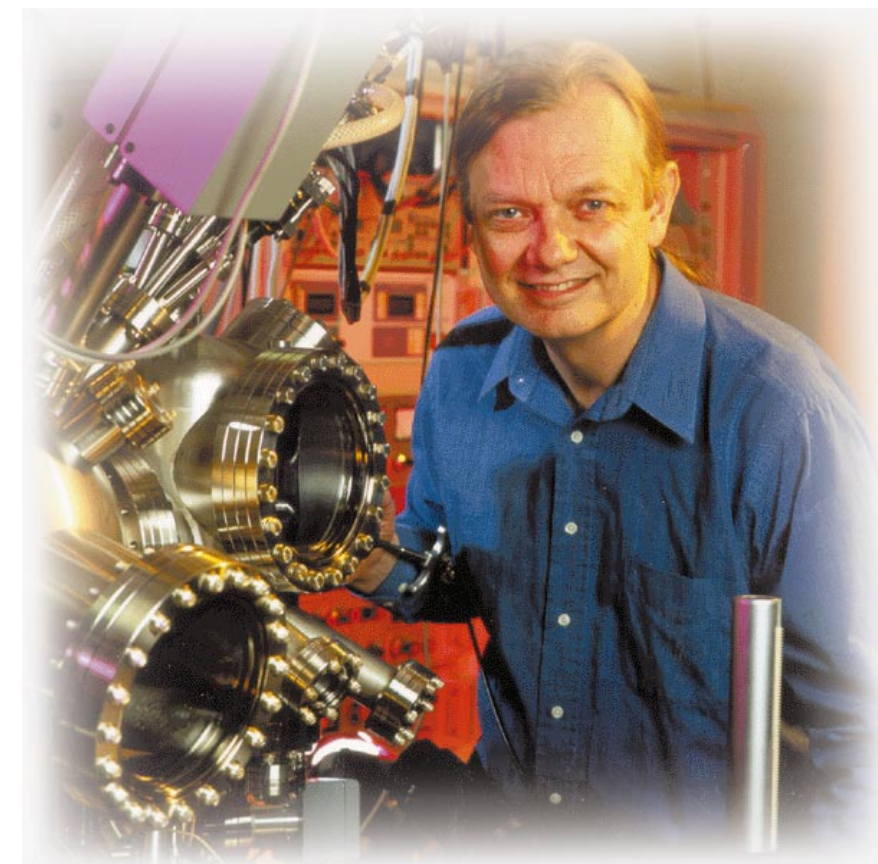
Silicon Valley is heading towards a brick wall. The development of ever more powerful microprocessors depends on continued progress in miniaturizing their components. But if current trends continue, conventional silicon chips will reach their physical limits around 2012. By then, their transistors will be so small that current leakage will become an insurmountable problem.

Until now, the industry's development has followed the famous rule of thumb identified by Gordon Moore, co-founder of the chip manufacturer Intel, which states that the number of transistors that can be packed onto a microprocessor doubles every 18–24 months (see figure, this page). The challenge ahead is to find an alternative to the silicon chip that will allow this rise in computational power to continue. Some researchers are placing their bets on quantum computing; others believe it will be necessary to process information by manipulating photons of light. But over the past few years, companies such as IBM and Hewlett-Packard have started seriously entertaining the idea of constructing computers in which computations are performed by individual molecules.

False start

Researchers have long been striving to create a viable 'molecular electronics' from molecules that act as linear wires, as transistor-like switches, and even as logic gates that process digital input signals. In 1974, Mark Ratner, now at Northwestern University in Evanston, Illinois, and Ari Aviram of IBM's Thomas J. Watson Research Center in Yorktown Heights, New York, set the ball rolling. They proposed that a molecule with a conjugated electronic structure — one in which single and double bonds alternate — could act as a rectifier¹. This basic electronic device allows current to flow in one direction only. Shortly afterwards, a team led by chemist Hideki Shirakawa, now at the University of Tsukuba in Japan, discovered how to make a form of polyacetylene that conducted electricity², raising hopes that single molecules of the polymer could act as wires.

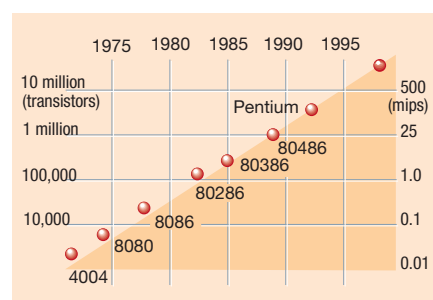
But subsequent progress in computing with molecules was frustratingly slow. In 1992, theoretical biologist John Hopfield, now at Princeton University in New Jersey, was moved to observe: "The field suffers from an excess of imagination and a deficiency



Chemical futures: Williams believes molecular logic will underpin tomorrow's computers.

of accomplishment." Yet in the past couple of years, the field's reputation has improved, boosted by the demonstration of molecular systems that can carry out the logic operations required of computer circuitry. That is a long way from a practical molecular computer. But it is proof of principle. "Molecular electronics offers a means for the information industry to continue the exponential increase in performance and lower cost for another two to perhaps even five decades," says Stanley Williams, an expert in nanoscale electronics at the Hewlett-Packard Research Laboratories in Palo Alto, California.

The first step on the road to molecular computing was to produce molecules that can be switched between two stable states, capable of encoding a binary 1 or 0 in a memory device. In the early 1990s, Robert Birge and his colleagues at Syracuse University in New York State demonstrated the principle



Moore's Law: the growing power of silicon chips, measured in millions of instructions per second.

by developing optical random-access memories (RAMs) based on films of the protein bacteriorhodopsin³. This molecule can be switched from one state to another by the absorption of light. But Birge's RAMs did not work at the single-molecule level — limits to the focusing of the laser beams used for writing and reading from the devices meant that many thousands of molecules in the film were switched in unison.

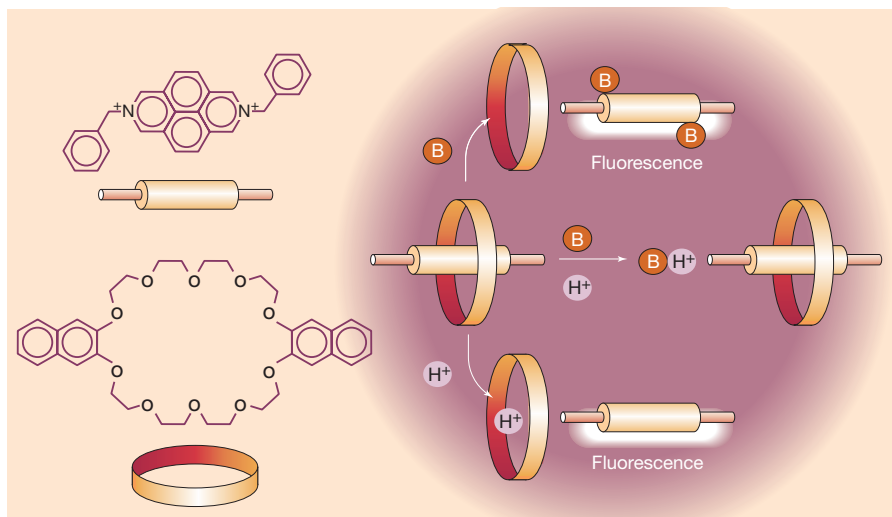
In England, chemist Fraser Stoddart's studies were yielding synthetic molecules that could act as switches. With his research groups at the University of Sheffield and later at the University of Birmingham, Stoddart developed techniques for making rotaxanes, which consist of a hoop-shaped molecule threaded onto a linear 'axle'. Bulky 'stopper' groups at either end of the axle keep the hoop from unthreading. In 1994, Stoddart and his colleagues made a rotaxane with an axle bearing two distinct 'donor' groups with which the hoop could interact. By oxidizing and reducing the molecules electrochemically, the hoop could be made to jump controllably from one group to the other⁴.

Making switches talk

To perform logic operations with such molecules, the switching caused by an input signal must be able to induce a change in an output signal. Only then might the switches 'talk' to each other or to other devices in a circuit. In most prototype molecular logic gates, the output is optical: an input signal alters the molecule's light emission or absorption properties.

As early as the 1980s, Birge devised molecular 'NAND' gates. These have two inputs and one output that fires (output 1) only when both inputs are activated (inputs 1,1). In Birge's molecules, two light-sensitive groups, or chromophores, influenced the absorption of light by an output chromophore by processes involving the transfer of electrons or energy within the molecules⁵.

More recently, researchers have created other varieties of logic gate. In 1998, a team led by Stoddart and Vincenzo Balzani at the University of Bologna in Italy created a molecular 'XOR' gate that used chemical input signals while producing an optical output due to the fluorescence of a chromophore. An XOR gate generates an output signal of 1 if the inputs are different (0,1 or 1,0), and an output of 0 if they are the same (0,0 or 1,1).



Light switch: the addition of an acid (H^+) or a base (B) to this pseudorotaxane allows the hoop to slip off the axle, which then fluoresces. When both the acid and the base are added together, the hoop remains in place and there is no fluorescence.

Stoddart and Balzani achieved this in a pseudorotaxane — a threaded hoop with no end stoppers, so that the hoop, which suppresses the chromophore's fluorescence, can slip off the axle (see diagram, above)⁶.

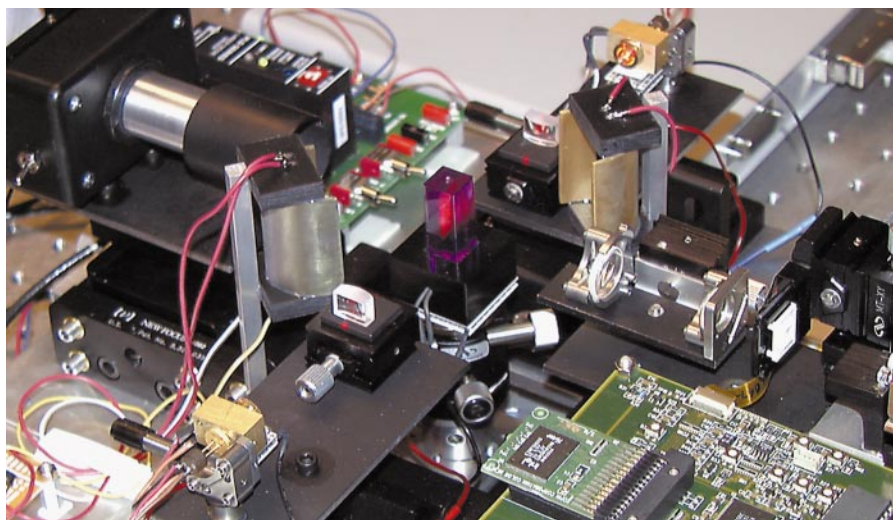
In April this year, chemists A. Prasanna de Silva and Nathan McClenaghan of the Queen's University of Belfast in Northern Ireland unveiled molecules with receptor groups that bind either calcium ions or protons, which together could do simple arithmetic⁷. The molecules carried chromophores with optical outputs that were altered by this binding. When both molecules were mixed in solution, their combined optical responses to the presence or absence of calcium ions and protons encoded binary responses to simple sums. For example, an input of '1,1' gave an output of '10', the binary equivalent of 2. While molecules that 'know' $1 + 1 = 2$ might not seem like much competition for silicon circuitry, they represent a significant step in the right direction.

Meanwhile, Balzani's team has described a photochemical system showing threshold behaviour, like that seen in nerve cells. This system consists of an organic molecule called 4'-methoxyflavylum and a cobalt complex, the absorption spectra of both being altered by light-driven reactions. A solution containing both molecules could 'integrate' two optical signals, 'firing' (absorbing light at a particular wavelength) only when these signals exceed a certain threshold⁸. This behaviour can only be achieved in conventional electronics by combining several components.

Simple molecular logic

All these examples involve molecules floating around in solution. De Silva suggests that their potential lies partly with the development of new chemical sensor systems. But solution-based molecular devices with optical outputs are of questionable value for complex information processing, because the emission or absorption of light is a general, multidirectional signal that does not readily allow one molecule to communicate specifically with another. "The significant problem we could never solve was how to connect the output of one gate to the input of another," says Birge of his pioneering 1980s work. That problem still dogs the field. "The chaining of one gate to another is ungainly, at best," de Silva concedes.

"Eventually, the molecular components of the system should be fixed in addressable arrays," concludes Balzani. This forces the issue of connectivity: how to wire them up. And that points towards electronics, rather than light or chemical diffusion, as the means of controlling the interactions between individual logic gates. Williams of Hewlett-Packard goes so far as to dismiss many of the demonstrations of molecular logic as "bogus," because the output signal is different from the input signal. "There is no



Molecular memory: this prototype RAM is based on the protein bacteriorhodopsin.

way to couple circuit elements together without transducers that would completely dominate the system," he observes.

What is needed, some researchers suggest, are molecules with electronic properties that mimic those of the workhorse of today's microprocessors: the silicon field-effect transistor (FET). This has three terminals: the source (input), drain (output) and gate (control) electrodes. In 1998, Cees Dekker and his colleagues at Delft University of Technology in the Netherlands created a rudimentary three-terminal transistor consisting of a carbon nanotube connected to two metal electrodes⁹. But the ability to wire up a single molecule like this is unusual, facilitated by the tubes being several micrometres long.

FETs do not merely control an output — they also introduce amplification, or gain. In theory, a molecule could do this job. In 1997, researchers led by Mark Reed of Yale University in New Haven, Connecticut, and James Tour of Rice University in Houston showed that the molecule benzene-1,4-dithiolate can transmit current across the junction between two gold electrodes¹⁰. Norton Lang of IBM's Watson research centre and his colleagues have recently shown theoretically that gain could be developed at this molecular junction, if a third, control terminal could be added¹¹ — although adding this is likely to prove extremely difficult in practice.

All the same, the field of molecular logic is starting to feel its way towards the fabrication of practical electronic devices. This was illustrated in dramatic fashion last year when Williams, working with Jim Heath and colleagues at the University of California at Los Angeles — including Stoddart, who is now based there — made solid-state logic gates based on rotaxanes¹².

With these molecules, the axle was kinked into a 'V' shape, and the switching did not seem to depend on motion of the hoop on the axle — instead, the molecules' electrical conductivity was switched by oxidizing the axle unit. The researchers first deposited a film of the rotaxanes onto an electrode. The

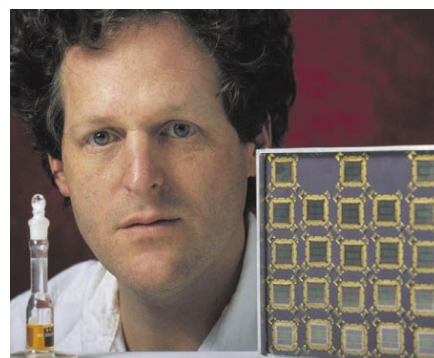
molecules in this monolayer were oriented so that the apex of each V pointed down at the electrode. They then deposited tiny metal wires on top of the rotaxane film. Applying a voltage to these wires oxidized the rotaxane molecules and irreversibly 'opened' the switch. Heath and his colleagues also showed that these switches could be connected into configurations that acted as two different varieties of logic gate. Each device comprised many thousands of rotaxane molecules. But in principle they could be scaled down to single-molecule dimensions, if electrodes could be made small enough.

Almost entirely memory

While such devices could form the basis of a molecular computer, the architecture of such a machine is likely to be quite different from that of today's microchips. "The big mistake that most people in this field are making," says Williams, "is to attempt to reproduce the functionality of discrete silicon devices in molecules, and then string the molecules together in the same way a silicon integrated circuit is built." Williams points out that the molecular switches he devised in collaboration with Heath's team are basically two-state memory elements. If you have enough memory, he says, computation can be largely done using 'look-up' tables rather than, as at present, calculating everything from scratch. "Computers based on molecular components will be almost entirely memory," Williams predicts.

Phil Kuekes, a colleague of Williams who specializes in computer architecture, says that the Hewlett-Packard team is already thinking about how to wire molecular memory arrays into nanometre-scale devices. Their approach borrows from the 1970s concept of 'programmable logic arrays', in which single chips using conventional electronic circuitry could perform basic logic operations, such as adding two bits together. Kuekes says the goal is to reproduce this function in molecular memory circuits measuring 100 nanometres across, which could be wired up using a grid of self-assembling nanowires with the arrays sandwiched at the crossing points. Significantly, Williams and his colleagues have just demonstrated the growth of such wires¹³.

As well as thinking more deeply about computer architecture, molecular logic researchers will need to address some fundamental obstacles inherent to the technology. For one thing, logic circuits consisting of arrays of molecules are sure to contain defects, since no chemical synthesis is perfect. But Heath and Williams argue that experience with the Teramac, a massively parallel but defect-ridden experimental computer built by Hewlett-Packard, suggests it should be possible to devise software routines to search for useable elements in an array containing many defective components¹⁴.



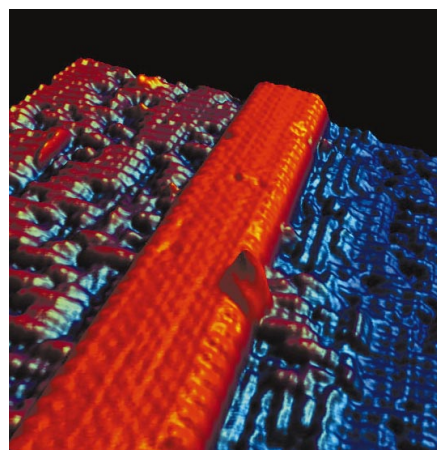
Heath: fabricating solid-state molecular gates.

At the scale of individual molecules, quantum effects might also limit reliability. In theory, molecular switches could operate incredibly quickly, with switching times measured in femtoseconds (10^{-15} seconds), rather than the nanoseconds of today's silicon FETs. At these fleeting timescales, the Uncertainty Principle will limit the precision with which the states of the switches can be measured — and thus the feasibility of storing and processing information. "This entire issue has been ignored in much of the recent work because such considerations appear to dampen the excitement," says Birge. But he believes it should be possible to counter the problem by applying some redundancy — encoding each bit of information in more than one molecule.

Aside from the technical obstacles, there is also the inbuilt inertia of the multibillion-dollar computer chip industry, which is understandably reluctant to abandon silicon for approaches that have yet to yield a single commercial device. But the developments of the past few years have convinced some within the computer industry that molecular logic will eventually deliver the goods. "I was one of the biggest sceptics," says Williams. "Now I believe that this is the inevitable wave of the future."

Philip Ball is a Consultant Editor of Nature.

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Nanowires could control molecular devices.

Landsat reveals China's farmland reserves, but they're vanishing fast

Sir— We have compared the official estimates of agricultural land and rates of agricultural land conversion with those derived from Landsat thematic mapper satellite images for 10 counties in the Pearl River Delta, which is one of the fastest-developing regions in China. Ground-based field assessments verify the high accuracy of our techniques in estimating the area of agricultural land and its change through time^{1,2}.

Our results indicate that there is significantly more agricultural land than reported in official statistics³. Although this under-reporting is well documented⁴, particularly using coarse resolution (1-km) satellite data sets⁵, our study is the first to use high-resolution satellite imagery to quantify this bias.

Satellite-derived estimates of total agricultural area in the ten counties for 1990 is 6,724 km²—this is 115% greater than the 3,119 km² reported in government yearbooks (Fig. 1a). Similarly, satellite-derived estimates for the total area of agricultural land converted to other uses between 1990 and 1996 is about 11% greater than the 789 km² reported in statistical yearbooks (Fig. 1b).

One interesting effect is that while the satellite-based method estimates more overall conversion of agricultural land, the fraction of the agricultural land converted is smaller than official estimates.

With the world's largest population and one of the fastest-growing economies, China's ability to feed itself has important domestic and international implications. Agricultural self-sufficiency is a concern because economic development is rapidly converting China's small per-capita stock of agricultural land to other uses^{6,7}. Analyses of the severity of this problem are based largely on official statistics for the stock of agricultural land and its rate of conversion, but these statistics may be biased by institutional factors.

Starting with the Great Leap Forward in 1958, Chinese farmers have had strong incentives to understate the extent of their agricultural land. Grain quotas were based on agricultural acreage, so understating agricultural area reduced the quota a farmer was expected to provide.

Although this production quota has been eliminated, farmers still have incentives to understate their agricultural land. A 1985 moratorium in the region limited the amount of agricultural land converted for other uses. This directive, together with a desire to evade taxes on land leased for

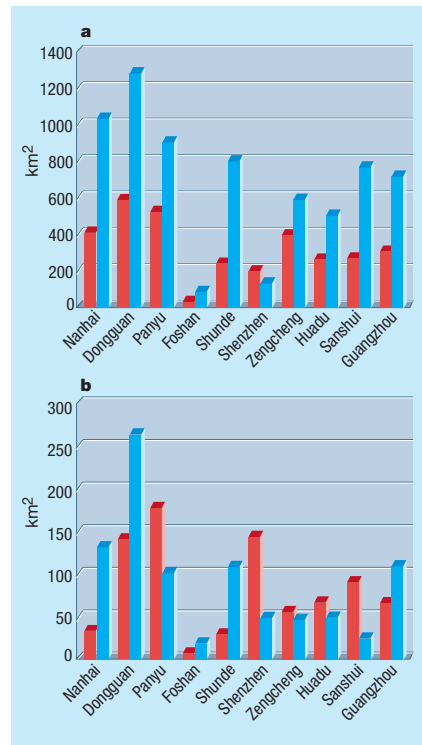


Figure 1a, Estimates of agricultural land in 10 counties of the Pearl River Delta, 1990, derived from satellite imagery (blue) and official yearbooks (red). **b**, Estimates of agricultural land conversion from 1990 to 1996 in 10 counties of the Pearl River Delta, derived from satellite imagery (blue) and official yearbooks (red).

commercial purposes, causes farmers to understate the area of agricultural land that has been converted to other uses.

Despite this under-reporting, we are not suggesting that the satellite-derived estimates are unbiased. Remote-sensing estimates may overestimate the amount of agricultural land because the resolution of the imagery (30 × 30 m) is too coarse to differentiate among small irrigation ditches, dirt paths and the other types of land use that co-exist with agriculture. Moreover, remote-sensing and government classification regimes of agricultural land may differ.

Our conclusion that there is more agricultural land than reported does not eliminate concern about the loss of agricultural land associated with rapid rates of economic development. Indeed, our results indicate that more agricultural land is being converted than reported.

Our results do, however, indicate that analysts must exercise caution when they use official statistics of agricultural land area and rates of loss to assess future rates of agricultural production in China.

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Please don't downgrade the sequencers' role ...

Sir— As representatives of the funding agencies that support the large-scale genome sequencing of parasites and related genomic research, we disagree with Colin Macilwain's description in the News report "Biologists challenge sequencers on parasite genome publication" (*Nature* **405**, 601–602; 2000) of the spirit of collaboration among the sequencers, funders and research scientists involved.

The data-release policy of these genome projects is for each sequencing centre to release unfinished sequence and assembled 'contigs' to a publicly accessible website. The policy encourages investigators to use the sequence data for gene-by-gene discovery. Already, in the case of the malaria parasite, more than a dozen peer-reviewed publications have used the preliminary sequence data and acknowledge the source of the sequences.

The sequencing centres and their collaborators need to keep responsibility for the computational analysis of unpublished sequence data on a chromosome or genome scale, for various reasons. First, the data are preliminary, and analyses will need correcting or completing as new data are added and existing data are modified. Second, the sequencing investigators and their collaborators should be allowed to confirm their research and publish the initial analysis of the data they have worked hard to generate.

Unfortunately, a few researchers view scientists at sequencing centres as service providers. In reality, they are conducting cutting-edge research, such as developing strategies for dealing with genomes with unusual base composition (*Plasmodium falciparum* is 80% A–T), including new methods to close difficult gaps in the sequence. In addition, they have designed and modified computer algorithms to identify and more accurately predict parasite genes and intron–exon boundaries.

Sequence data are no different from other types of biological data, and their generators deserve the same rights—to do an initial analysis and to publish the results—that other researchers have traditionally enjoyed. These data and their analysis should be published in peer-reviewed journals as quickly as possible. Until then,

the sequencing centres and their collaborators are seeking ways to provide added value to the unpublished sequence data.

At a meeting of the malaria genome project in June at the Wellcome Trust Genome Campus in Hinxton, UK, the sequencing centres agreed that a first-pass annotation would be added to their websites, including posting the results of blast hits for all the open reading frames. Participants in the malaria genome project have agreed on the development of the PlasmoDB database (<http://plasmodiumdb.cis.upenn.edu>), a web-accessible relational database facilitating the analysis of various genome-related data. With support from a grant from the Burroughs Wellcome fund, this database is being developed by David Roos and his colleagues at the University of Pennsylvania and by Ross Coppel at Monash University.

Contrary to your News report, the PlasmoDB team is not supported for, or engaged in, any effort to annotate the genome. Discussion is under way, however, to determine the extent of automated annotation that will be provided through PlasmoDB for unfinished sequence available from the sequencing centres.

WHO's programme for research and training in tropical diseases (see below) is to provide support for a 'help desk', in addition to the current distribution of data through a CD-ROM version of Malaria Information Resource, to help investigators who lack the computational resources to access and analyse the data.

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||UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), 1211 Geneva 27, Switzerland

... when public-interest science needs solidarity

Sir — As a member of the small community of people working on malarial parasites, I am a constant user of sequence databases from the consortia at Sanger Centre, TIGR and Stanford (*Nature* **405**: 601–602; 2000). I am deeply grateful to these centres, which release all the data freely on the Internet, even in raw form, as soon as they are available.

According to the data-release policy of these three consortia, “these data will assist colleagues in their research, particularly in the search for genes and the studies of the genes’ biological function” and “provide investigators with information that may jump-start biological experimentation”. New perspectives are being added to virtually all the ongoing research projects and many more projects will start, as well as other ‘brute force’ approaches such as roteomix and SAGE (serial analysis of gene expression). All these data together will be integrated by bioinformaticists into more sophisticated and complete databases.

I sympathize with those who say that a period of “non-hypothesis science” has started, but I don’t agree with them. Good scientists will go on making good hypotheses, and the vastness of available data will only eliminate time-consuming and painstaking benchwork. As things stand, much laboratory work contributes to the efforts of the sequencers by providing clues for annotation and function. In the near future, many other hints will help to clarify the fine structure of the sequenced regions, for example when introns and exons are present that are too small to be revealed by the currently used algorithms, or when regulatory regions need to be identified.

For these reasons, I found the quarrel between the sequencing consortia and laboratory researchers reported in your News article very worrying. It would of course be wrong for a tribe of ‘annotators’ to develop, exploiting raw data only for their own publications instead of helping the sequencers. But, again, the release policy states that any investigator should ask permission of the consortia to publish data based on the content of the databases, and referees should easily be able to distinguish between a genuine contribution and data piracy. In this regard, the PlasmoDB database of Roos and colleagues represents an important tool for all of us and cannot be described simply as an “Internet portal” or connected with the concept of “piracy”.

Scientific careers are largely based on the amount and quality of publications, but it would be impossible for every new chromosome of every organism to be published in the highest-impact journals such as *Nature* and *Science*. Perhaps an online journal or supplement dedicated to sequencing and annotation work should be launched.

The most serious aspect of this situation is, however, not mentioned in the malaria article but is well put in another News report on the same pages: “Drive for more genomes threatens mouse sequence” (*Nature* **405**: 602–603; 2000).

Craig Venter of Celera Genomics is quoted as stating that the mouse genome is almost correctly assembled, directed by the blueprint of the human genome — that is,

by fundamental work done by public enterprise and non-profit consortia. In spite of this, Venter says Celera’s mouse genome will be restricted to subscribers.

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The search continues for Kármán’s St Christopher

Sir — It is nice to see fourteenth-century Bolognese frescoes getting a bit of well-deserved publicity in the Science in Culture article “St Christopher and the vortex”¹. However, if Theodore von Kármán was inspired by the beautiful San Domenico fresco of St Christopher to conceive of his ‘vortex street’, his powers as a psychic would be at least as remarkable as his achievements in the physical sciences.

True, the fresco in the church of San Domenico was painted during the late fourteenth century (to a formula much used by Tomaso da Modena and his associates²) and Kármán was born in 1881. But it had been hidden since 1728–31 behind a staircase built up to an organ loft. It was only revealed during repairs in 1979.

So what was the painting that Kármán reported had inspired him? Mizota and his partners are surely correct in seeing fourteenth-century painting style in Kármán’s water-flow representation, but today remarkably few examples of St Christopher survive in Bologna itself: the grandest, in San Petronio, by Giovanni da Modena (circa 1410–20), has been cut off at the ankles. Tomaso da Modena painted a similar image in San Francesco, Treviso, around 1350. A pupil of his painted another in the next chapel; similar accumulations probably occurred in churches in Bologna, but were lost during the Counter-Reformation.

The representation of St Christopher that Kármán is most likely to have seen is the triptych by Jacopo di Paolo from the 1390s that has long been shown in various rooms of the outer cloisters of San Stefano³ — but this comes nowhere near the San Domenico fresco in the clarity with which it shows water flowing.

We might deduce from this that von Kármán was sufficiently sensitive to painting style to have read the appropriate patterns into what he saw, from his own knowledge of both art and water, in his moment of inspiration.

Robert Gibbs

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1. Mizota, T. et al. *Nature* **404**, 226 (2000).

2. D’Amico, R. *Strenna Storica Bolognese* **33**, 95–113 (1983).

3. Arcangeli, F. *Pittura Bolognese del '300* (Grafis, Bologna, 1978).

Quantum weirdness

How much of the strangeness lies in Feynman's legacy of catchphrases?

The Strange World of Quantum Mechanics

by Daniel F. Styer

Cambridge University Press: 2000. 154 pp.

£14.95, \$24.95 (pbk)

Peter Holland

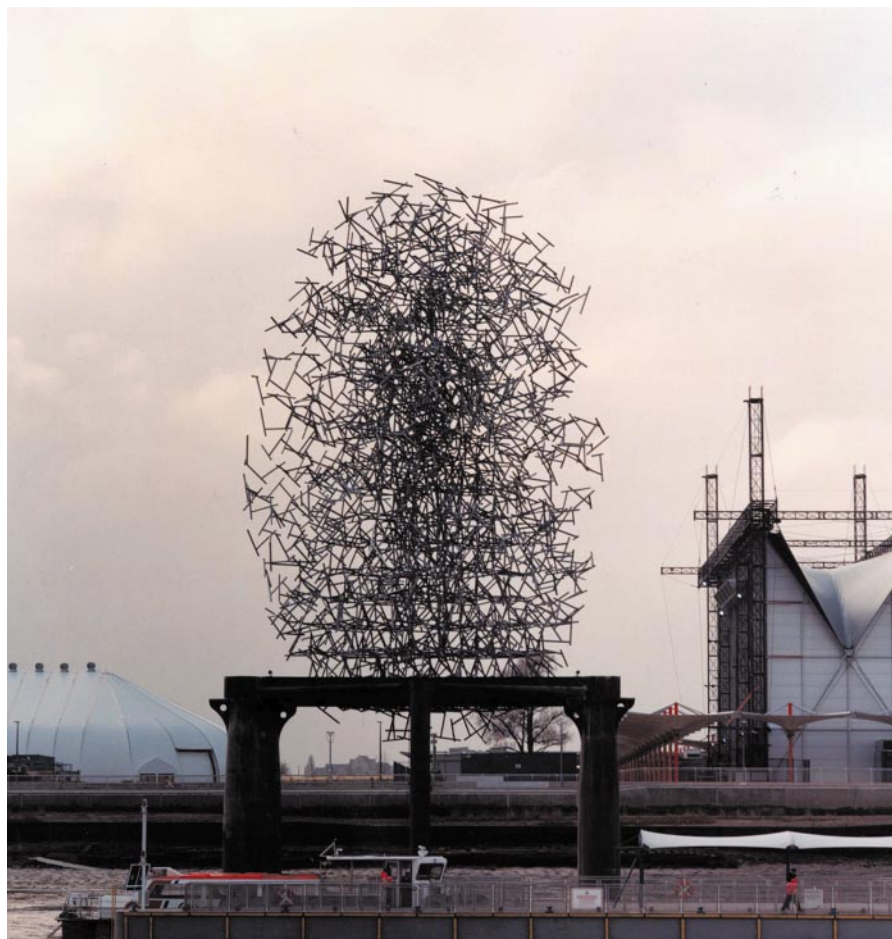
The proposition that the world is so weird that it defies common sense has always been a major theme in popularizations of quantum mechanics. Generally, the claim is self-fulfilling — the subject is presented in such a way as to make this conclusion inescapable. Leaving aside the commercial opportunities afforded by the New Age market, these popularizations reflect the dominant view of professional physicists since the genesis of quantum theory in the 1920s.

The current generation of writers is heavily influenced by Richard Feynman's pragmatic approach to interpretation. Since his death, collections of Feynman's writings have appeared more frequently than Beach Boys compilations, providing a rich source of ready-made catchphrases ("Nobody understands quantum mechanics"). Two Feynman works, in particular, set the agenda for Daniel Styer's book: *The Character of Physical Law* (BBC Publications, 1965), which sets up the conceptual conundrum and asserts a primary role for probability, and *QED* (Princeton University Press, 1985), in which the task of computing probabilities is translated into pictorial terms. Like Feynman, Styer addresses a non-technical audience.

In this approach, physical processes are 'black boxes' — they have inputs and outputs, and the weirdness arises because, supposedly, these are not causally connected. Quantum mechanics is then not a physical theory but an abacus for computing the relative probability of outputs.

Styer believes one must master this 'standard view' before considering alternative views. This is problematic from several standpoints. Traditionally, the standard view has been associated with Niels Bohr and Werner Heisenberg, but Styer dissociates himself from that lineage. So the standard view itself has no unique formulation. Moreover, given that alternatives exist, the author does not explain what singles out the 'standard' one as that preferred. After all, no one adheres to black-box models in practice; as the author admits, physicists instinctively make informal models of the world.

Within its terms of reference, the book gives a clear account of Feynman's approach. At times this is quite compelling, as in the



Quantum Cloud: Antony Gormley's sculpture was created using, among other tools, chaos theory.

treatment of the quantum bouncing ball. One valuable idea that I haven't seen before in this kind of book is the inclusion of challenging problems at the end of each chapter.

Styer is forthright in pointing out defects in other popular works, but his own analysis is at times questionable. For instance, he tells us that we mustn't dictate to nature, yet in several places declares that an electron does not have a trajectory. How does he know this? Certainly, nothing in quantum mechanics or in Styer's presentation dictates this conclusion. There is, after all, a perfectly consistent view of quantum mechanics — that drawn up by Louis de Broglie and David Bohm — which does attribute a trajectory to an electron. Styer actually alludes to this theory several times (although he does not give a reference for it, which is strange, as his other referencing is excellent), but dismisses it as weird. What a weird criticism, when one's aim is precisely to establish weirdness!

I wonder what Styer's student readership

would have thought had he reproduced the iconic figure of Bohm's trajectories for the two-slit experiment. My experience is that they would have asked why this model isn't fully explained in books such as this one, and, indeed, why it isn't the 'standard view'. To be sure, Bohm's theory has unresolved problems (fertile theories do). But the point is that it allows one to analyse how the black box functions through a causally connected sequence of events from input to output. As probability is no longer the basic concept, this view is more complete than the standard one, with no need to invoke images of "shimmering colours" when referring to electrons. Surely, students should be told of this.

If you are looking for an original account of Feynman's approach, I recommend this book. But surely the weirdness card has been played enough by now — isn't it time to do it differently?

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Genes, free will and intracranial musings

Alas, Poor Darwin: Arguments against Evolutionary Psychology

edited by Hilary Rose and Steven P. R. Rose
Jonathan Cape: 2000. 242 pp. £17.99

David L. Hull

The major theme of this anthology is that the work of evolutionary psychologists, the lineal descendants of sociobiologists, is too simplistic to handle the incredibly complex phenomena they purport to explain. These critics round up the usual suspects (such as E. O. Wilson, Richard Dawkins, Dan Dennett, Helena Cronin, John Tooby and Leda Cosmides) and raise familiar objections to the usual conceptual culprits (genetic determinism, reductionism, gene selectionism, Panglossian adaptationism, and so on).

As always, the authors involved in these disputes caricature one another's views but object to being caricatured themselves. For example, Dawkins claims that genes are self-replicating entities. Steven Rose spends several pages arguing that genes are not literally 'self' replicating — for starters, all sorts of additional cellular constituents are needed — as if Dawkins thought that a strand of DNA all by itself on a glass slide might suddenly start replicating. Rose admits that Dawkins knows all this, but goes on to complain that the tenor of Dawkins' writing leads his readers to ignore these other constituents.

Authors engaged in controversies such as this one are seldom charitable in interpreting each others' prose. In addition, they seem to vacillate between strong and weak versions of their views. Genes are the fundamental units of replication. Genes are the fundamental units of selection. All there is to selection is replication. Misrepresentation and equivocation over precisely what is meant are so common that one need not be a Panglossian adaptationist to suspect that such practices are doing some good. One 'just-so' story is that it helps authors to clarify their ideas as they reformulate them under the pressure of being misconstrued. Regardless of our desire, few of us know precisely what we mean when we are developing new ideas. Seeing what others have taken us to mean forces us to re-evaluate our earlier pronouncements.

The critics of evolutionary psychology are also of two minds when it comes to such just-so stories. Gabby Dover complains that in "modern-day story-telling we are free to imagine any ecological 'lock' in the distant days of hunting and gathering by our proto-human ancestors to which the 'key' of contemporary behaviour (such as sexuality, aggression, fear, parental investment) has been selectively fashioned". But then, these critics of biological just-so stories provide

sociological stories that are even more problematic. Rose and Rose ask in their introduction why evolutionary psychology has become so popular in recent decades. Their answer is that it is the result of the "search for new apparent certainties" as "old seeming certainties and indeed hopes for a more socially just future have crumbled". I fail to see how that is any less of a just-so story than the ones the book's authors decry.

The basis of many objections to evolutionary psychology is the ancient philosophical problem of free will. Biological (genetic) determinism seems to preclude human beings, at least occasionally, from acting freely: 'It's not my fault; my genes made me do it'. Instead, they emphasize the role of the environment. But environmental determinism is no less deterministic: 'It's not my fault; my environment made me do it'. The next step is to recognize that genes and environmental variables are intimately intertwined: 'It's not my fault; my intimately intertwined genes and environment made me do it'.

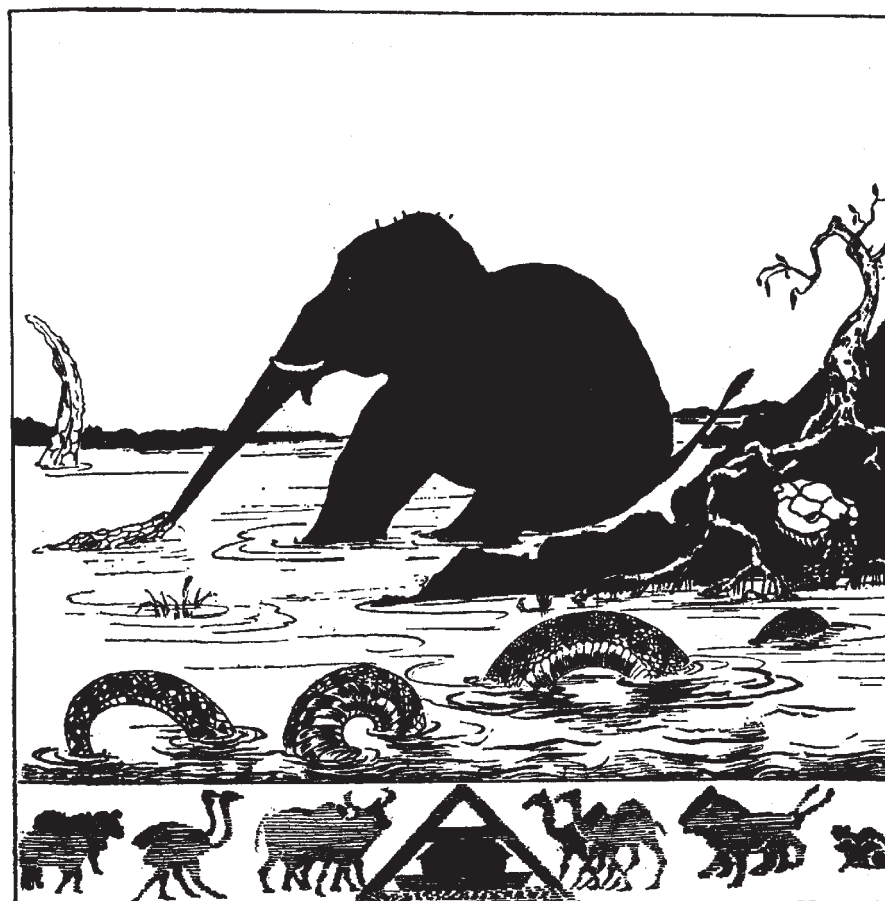
Although a desire to establish the existence of free will seems to motivate several authors in this book, none of them presents much in the way of a satisfactory defence of free will. Nor have professional philosophers over the past two millennia been any more successful. To paraphrase one philosopher, free will is not only compatible

with determinism but impossible without it.

A minor theme of this anthology is post-modernism, represented by a hodgepodge of views that emphasizes the power of language and downplays the sort of knowledge provided by science — "facts are socially constructed". At one extreme, this claim can mean that we can construct the empirical world in any way we see fit. Having paralysed legs is a 'disability' only if one defines it that way. A more reasonable position is that, in the vast majority of human environments, having paralysed legs is certainly an impediment, but disabling environments also play a central role. As we design buildings and sidewalks to accommodate wheelchairs, having paralysed legs becomes less disabling.

Charles Jencks' paper is the only contribution that is post-modern throughout. One of the strengths of post-modernists is that many of them write entertainingly. After all, many of the elements of post-modernism came from literary criticism. In spite of myself, I enjoyed Jencks' intracranial musings as he sat through a paper being delivered by E. O. Wilson — or "Mr. Darwin", as Tom Wolfe baptized him. His remarks are playful, but have bite. I also enjoyed Mary Midgley's paper, but for the opposite reason; it was measured and considerate.

Rose and Rose's introduction claims that the anthology combines criticisms of



How did the elephant get its trunk? But just-so stories extend well beyond Kipling's collection.

MARY EVANS

evolutionary psychology with the development of alternative explanatory perspectives. However, the book's subtitle is "Arguments Against Evolutionary Psychology", and the contributions in this anthology are primarily critical in character. Nothing like the detailed, highly confirmed theories that the book's contributors demand of evolutionary psychologists can be found in these pages for 'alternative perspectives'. We get hints, and explanations of why we are given only hints, but that is it. The phenomena are all too complicated to do more. The mistake evolutionary psychologists make is to provide simplistic explanations for such complex phenomena.

A famous historian of science claimed in 1950 that hereditary phenomena are so complicated that no molecule could ever do what genes are supposed to do. A couple of years later, James Watson and Francis Crick came up with an amazingly simple molecule. The immune system is extremely complicated — so complicated that I cannot claim to understand it very well — but it is there to be understood, no matter how complicated it might be. Rose and Rose remark that "bad theory can never be driven out solely by criticism". If so, the critics of evolutionary psychology could make better use of their time by developing these alternative theories, no matter how complicated they turn out to be. Repeating overly familiar criticisms of evolutionary psychology and sociobiology is unlikely to have much effect. For all their crudity and lack of sophistication, evolutionary psychologists keep churning out book after book, paper after paper, both popular and technical. They are not content to carp on the sidelines. ■

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Crushed by the Frankenstein monster

**Inventing Ourselves Out of Jobs?
America's Debate over
Technological Unemployment,
1929–1981**

by Amy Sue Bix
Johns Hopkins University Press: 2000.
376 pp. \$45, £35

Howard P. Segal

On 1 February 1933, at the height of the Great Depression, the *New York Times* published a front-page interview with car-maker Henry Ford. As unprecedented numbers of Americans endured economic catastrophe, Ford proclaimed that these were "not bad times but good ones", and that the nation was "on the threshold of an inconceivably bright future". Ford based his seemingly perverse position on two principal points: first, the



Down and out: unemployed workers demonstrate in 1932 over 'technological unemployment'.

Depression was steadily cleansing American business of its accumulated laziness, inefficiency and technological obsolescence, even within the automobile industry; and second, the so-called contemporary 'Machine Age' was just beginning and would eventually create unparalleled long-term prosperity.

Ford took particular exception to the growing notion that machines of all kinds were putting ever more people out of work and that 'technological unemployment' — as this phenomenon had begun to be termed — was the wave of the future. "It is nonsense," Ford argued, "to call the machine a Frankenstein monster which is crushing its creator." On the contrary, the Ford Motor Company's "experience for thirty years is that every time we reduced the number of men on a given job, and thus lowered costs, we had to hire even more men on account of increased business". Equally important, the machines that brought about such changes "must be built by other machines, and it takes men to build them and other highly paid men to design them". As the Machine Age evolved, there would be ample employment for all, including women.

Amy Sue Bix's impressive book provides a rich historical and contemporary context for comments such as Ford's. No historian before her has examined systematically what she rightly calls the American debate over the role of machines in either reducing or increasing jobs. Far from being decided by the early 1980s, moreover, that debate continues in our high-tech era, as Bix makes clear.

But Bix's focus is the 1920s and '30s, when, for the first time in American history, the public made inventors, scientists and engineers as responsible for economic bad times as greedy industrialists. Previously, only the Andrew Carnegies and John D. Rockefellers had been condemned. The Thomas Edisons and George Westinghouses had not only escaped any blame but had been elevated to hero stature. Now, however, even President Herbert Hoover, long praised as the "Great Engineer", was not spared. The very technological expertise he had brought to government service years before, as a distinguished mining engineer and then visionary social engineer, now haunted him as he proved incapable of alleviating, much less preventing, America's greatest economic crisis.

Still, as Bix observes, the United States never witnessed anything comparable to England's eighteenth-century Luddites, or 'machine breakers'. Most workers who lost their jobs simply resigned themselves to the supposed inevitability of technological 'progress'. Nor did union leaders condemn the technological developments behind these job losses. Instead, they wished only to ensure that workers who kept their jobs shared in whatever profits mechanization provided.

Bix also discusses the depiction of technological unemployment in Depression-era popular culture, and its manifestation in radio and movies, high-brow literature and science fiction, jokes and cartoons. And she persuasively reinterprets the 1939–40 New York World's Fair as being as much a calculated defence against fears of technological unemployment as a celebration of the "World of Tomorrow".

Nevertheless, Americans' historic bedrock faith in progress through technology — and their traditional equating of technological progress with social progress — were seriously challenged by the spectre of technological unemployment. But that faith survived. Still, the very phrase 'Machine Age', which dates back at least to the late nineteenth century, lost for ever its strictly positive connotation. The seeds of the widespread disenchantment with technological advances that have sprouted since the 1960s were, I believe, sown in this Great Depression debate.

Similarly, Bix's work can be linked with the technological determinism that contemporary high-tech takes for granted: the conviction that technology shapes everything and that all else in the world must accommodate it. Bix describes how many scholars in the 1920s and '30s viewed technological unemployment as the almost inevitable result of American society's inability to cope with the economic, social and psychological effects of unceasing technological advance. Sociologist William Ogburn termed this phenomenon "cultural lag", and — decades before the high-tech prophet Alvin Toffler — used the metaphor of the wave to suggest the futility of resisting technological advances.

Although Bix's book mentions neither Toffler nor any of the other high-tech visionaries who routinely embrace technological determinism, her work could profitably be read as an antidote to theirs. Like every other historian of technology, Bix recognizes that technological determinism is historical fantasy. Yet her analysis of Ogburn and his fellow believers in cultural lag is as scrupulously fair to all viewpoints as is the rest of her book.

Inventing Ourselves Out of Jobs? is a first-rate historical study that simultaneously speaks to our high-tech present. It could not be more timely or more deserving of a wide readership. ■

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Shelling out the facts

The Ecology of Freshwater Molluscs

by Robert T. Dillon Jr

Cambridge University Press: 2000. 509 pp.

£75, \$120

David L. Strayer

Molluscs have traditionally taken a back seat to insects and crustaceans in freshwater ecology, and rarely feature prominently in courses or textbooks on the subject. Yet recent work shows that suspension-feeding bivalves and grazing snails can control the abundance and composition of primary producers such as phytoplankton, and thus have far-reaching impacts on freshwater ecosystems.

Freshwater snails are intermediate hosts for important pathogenic trematodes of humans and livestock, thereby occupying a central place in what might be called medical limnology. Moreover, as hundreds of species of freshwater molluscs are extinct, or imperilled by human activities, the group urgently needs the attention of conservation biologists. In other words, there are good reasons to bring molluscan ecology closer to the ecological mainstream.

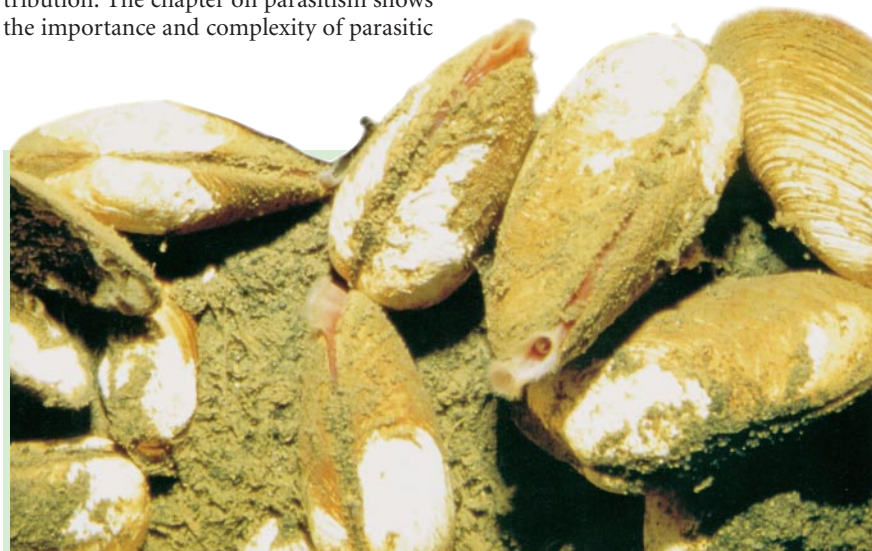
Rob Dillon believes that freshwater molluscs have their own special ecology, distinguished by their immobility, indiscriminate diet and high requirement for calcium. He also thinks they have a largely untapped potential to contribute to the advancement of general ecology, and his book covers their life history, diet, biotic interactions and distribution. The chapter on parasitism shows the importance and complexity of parasitic

interactions, and will be especially valuable to ecologists, who tend to overlook parasitism as an important ecological factor. The book also contains original analyses of published data, and proposes a new framework (based on J. Philip Grime's triangular model) in which to interpret molluscan life histories and distributions.

The chief strength of this book is its presentation and analysis of a large, scattered body of literature on freshwater molluscs. The literature coverage is impressively wide and international (but not very multilingual), although some key studies are inexplicably missing. Similarly, several research topics that have figured prominently in freshwater molluscan ecology (for example, the ecological roles of molluscs and their historical biogeography) are poorly represented.

Dillon's analyses are alternately stimulating and irritating. I jotted down many ideas for new research projects in the margins of the book. At the same time, I feel that many of Dillon's conclusions are premature or even incorrect. Further, the narrow focus on molluscs has caused him to miss opportunities for making connections to the broader ecological literature on topics such as the selection of food items by herbivores and sublethal effects of predators on prey. Thus, this book should be read critically. Despite these flaws, however, it is a valuable resource for students seeking fresh research questions, readers looking for information about freshwater molluscs, and ecologists who want to see what freshwater molluscs have to offer. ■

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All clammed up

Bivalve molluscs tend to cluster in areas of the Monterey Canyon where sulphide-rich water percolates up through the sediments.

From *The Deep Sea* by Bruce Robison and Judith Connor (Monterey Bay Aquarium Press, \$9.95, £8.95).

The structure of matter

Like science, teaching should be the result of independent ideas converging.

Harry J. Lipkin

The twentieth century began with the consensus that matter is not continuous but is made up of atoms and molecules. It ended with the confirmation that matter is made of even tinier objects called quarks. But how are such realities established? And how can the process be explained in a plausible way to a non-scientist? I was surprised to find the answer in a book, *The Schools We Need and Why We Don't Have Them* (Doubleday, New York, 1996), by E. D. Hirsch Jr, a professor of English at the University of Virginia.

I read this book because of my concern about the large numbers of functionally illiterate children in both Israel and the United States unnecessarily condemned to the lowest levels of a democratic society. Professionals in the field of reading education are still obsessed with fighting wars between outdated 'ideologically and politically correct' party lines called phonics and whole language, rather than using new knowledge obtained from research and classroom experience to teach children to read. How to use this knowledge to provide effective education is well documented in extensive research sponsored by the US National Institutes of Health.

Twenty years of experience in Israel have shown how all this knowledge and information can be combined to produce a system that can teach 95% of a heterogeneous class of as many as 40 six-year-old pupils to read, to understand written texts, and to become independent learners with a single teacher and no help from parents. Not only does Hirsch's book provide the theoretical background supporting the empirical success of this system, he also gives an excellent description of how the scientific community reaches conclusions.

He writes: "The pattern of independent convergence (a kind of intellectual triangulation) is, along with accurate prediction, one of the most powerful confidence-building patterns in scientific research. There are few or no examples in the history of science (none that I know of) when the same result, reached by three or more truly independent means, has been overturned."

He quotes Abraham Pais's biography of Einstein for an example of this convergence: "The debate on molecular reality ... was settled once and for all because of the extraordinary agreement in the values of N (Avogadro's number) obtained by many different methods. Matters were clinched

not by a determination of N but by an overdetermination of N . From subjects as diverse as radioactivity, Brownian motion, and the blue in the sky, it was possible to state, by 1909, that a dozen independent ways of measuring N yielded results which lay between 6×10^{23} and 9×10^{23} ."

In 1966, Richard Dalitz and I were both already convinced that matter was made of quarks when we led the discussion on this topic at the annual international conference on high-energy physics. We could not understand why this conclusion was not generally accepted until well into the 1970s. I now realize that we were experiencing Hirsch's independent convergence. Unexplained regularities in the spectrum of particles created in high-energy accelerators; simple relations between collisions among different kinds of particles; that the annihilation of a proton and an antiproton

at rest nearly always produced three mesons; relations between the electromagnetic properties of mesons and baryons; and the 3/2 ratio of the magnetic moments of the neutron and proton all converged on the same conclusion: mesons and baryons are built from the same elementary building blocks. Hirsch applies this concept to pedagogy: "The independent convergence on the fundamentals of effective pedagogy that exists today is less mathematical but nonetheless compelling."

I find Hirsch's demonstration of how the scientific community reaches conclusions and of how the results of research on reading can be interpreted very exciting. I have always disliked the explanations of the 'scientific method' presented by social scientists in which scientists are said to do experiments to 'test hypotheses'. The description of the acceptance of new ideas as a result of independent convergence fits the reality that I know.

The gap between natural and social scientists is much too large and deserves serious attention. Perhaps these ideas can stimulate new lines of communication to overcome this gap. Perhaps they can also direct us to using new knowledge in finding better ways to teach children to read.

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The gap between social and natural scientists is much too large and deserves serious attention.



School's in: 95% of six-year-old Israelis can read, understand and learn independently.

The Oort crowd

The gods truly are moving in mysterious ways.

Ken MacLeod

As we enter the first year of the 23rd century (or the last year of the 22nd — some arguments never go away) we look back with satisfaction at the triumphs of science and technology in the first two centuries of the third millennium. The advances in medicine, in biotechnology, in communications, in atmospheric engineering have been more than adequately celebrated elsewhere. They are familiar to the most isolated farmer on the barest rocks of Antarctica. But in long-term significance for the human prospect, nothing can compare to the discovery of the gods.

The word 'gods' is used advisedly. Humanity's earliest speculations about the nature of any superhuman intelligences with which it might share the Universe are, paradoxically, more relevant to our real situation than the predictions of alien contact in the once-popular genre of science fiction. It must be admitted, however, that some of its practitioners (see, for example, Boyce, 1998, <http://www.et-presence.ndirect.co.uk>) reached part of the truth.

That truth, as we all know, is that a large, undetermined and (for good reason) indeterminable fraction of the bodies in the asteroid belt, the Kuiper Belt and the Oort Cloud are the sites of complex intelligent life. The precise evolutionary route(s) from extremophilic microorganisms to intelligence, apparently bypassing multicellular organization, remain unknown and perhaps (again, for good reason) unknowable. Computer simulations have yielded interesting, if inconclusive, results (Chang-Hoskins, 2197, provides a useful overview).

Those of our readers who have benefited from advances in medicine may recollect, and our younger readers can easily retrieve, the excitement that greeted the initial, accidental discovery of an ET intelligence in 2031. The first downloads from the Gates Foundation asteroid prospector revealed, not the potential wealth of resources expected in a carbonaceous chondrite, but a complex interior structure variously described as 'crystalline', 'fractal' and 'organic'. Fortunately for scientific openness, the drilling operation was webcast live, and as the pictures slowly scrolled down the screens of a few hundred thousand space enthusiasts, the news spread across the net faster than a virus.

In those first hasty, misspelled e-mails and postings we can see — from references to the

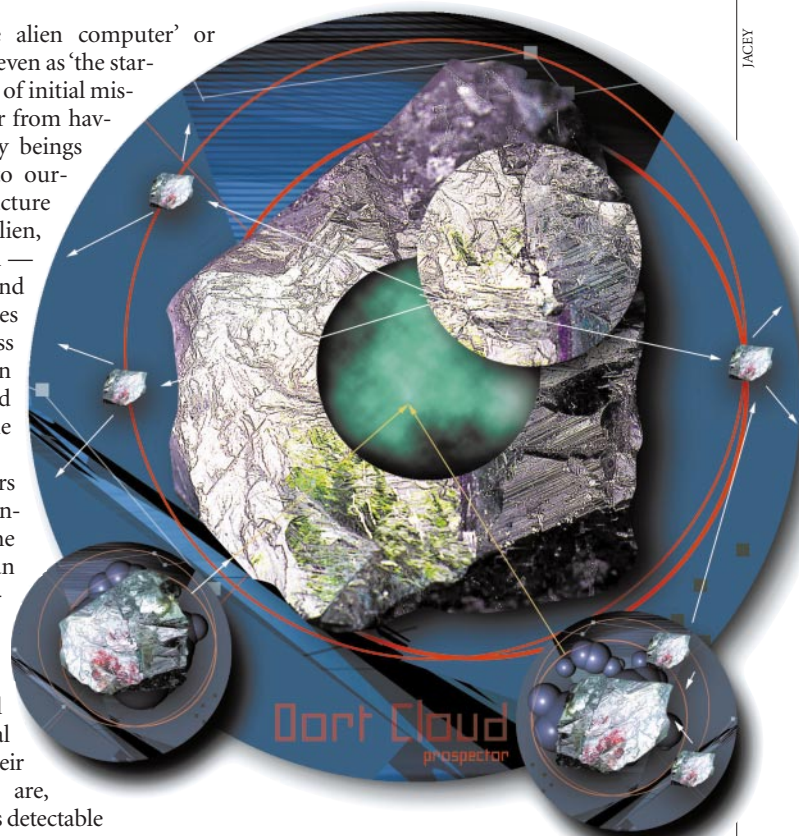
structure as 'the alien computer' or 'Asteroid City' or even as 'the star-ship' — the depth of initial misapprehension. Far from having been built by beings broadly similar to ourselves, the structure itself was the alien, or the civilization — the nature and number of centres of consciousness within it remain controversial. And it was neither alone nor isolated.

Billions of years of evolutionary 'tuning' have given the cometary minds an exquisite sensitivity to the electromagnetic output of each other's internal chemical and physical processes. Their communications are, once looked for, as detectable as they are incomprehensible. Some of the larger bodies in the Oort seem to act as relays, extending the communications net across solar and possibly interstellar distances. (As is known, the tenuous outer reaches of the Oort Cloud intersect those of their Centaurian equivalent.)

Despite strenuous efforts, no human communication with the extraterrestrial minds has been established (the results claimed by Lunan, 2049, are at best ambiguous). They are, to us, in precisely the position of the gods postulated by Epicurus, serene in the spaces between the worlds.

These gods, while indifferent, are not passive. Subtle control over their outgassings results, over very long periods, in orbital changes. More rapid processes occur within the asteroids. Careful study of recent and historical Near-Earth Objects suggests that the orbits of at least some NEOs have been the result of conscious intent.

In view of the above, it appears in retrospect unfortunate that the first probe to the Oort Cloud and beyond, launched in 2030, should have used as its initial means of propulsion a plasma sail consisting of ionized gas within a 'magnetic bubble' thousands of kilometres across, and as its secondary means a prototype 'electromagnetic ramscoop' sucking in vast quantities of



interstellar and cometary matter. Subsequent changes in the volume and intensity of intercometary communication, and in the orbits of numerous comets and asteroids, cannot be accounted for by the physical effects of its passage. They can only be considered a response.

The effect on human society of the discovery of the gods has been positive. Excluded from many of the space-based resources once thought unoccupied, we turn to a less profligate use of our planet's own. The expectations of John Stuart Mill, in his famous chapters on the "stationary state" and "the probable futurity of the labouring classes", have been largely realized.

But, as our astronomical and space-defence workers' cooperatives continue their urgent sky-watching, there may be some risk of overlooking a danger closer to home. There is no reason to suppose that extremophilic consciousness is confined to minor interplanetary bodies. Perhaps the majority of the Earth's biomass consists of subterranean extremophiles.

Watch the ground.

Ken MacLeod is the author of *The Star Fraction*, *The Stone Canal*, *The Cassini Division* and *The Sky Road*. His next novel, *Cosmonaut Keep*, expands on some of the ideas in this article.

Segmentation *in silico*

Peter Dearden and Michael Akam

A new mathematical biology is emerging. Building on experimental data from developing organisms, it uses the power of computational methods to explore the properties of real gene networks.

Life depends on the interaction of tens of thousands of genes and their protein products, orchestrated by the regulatory logic of each genome. If we are to comprehend this logic, we must hope that it can be dissected into a series of interlinked modules or networks, each of which can be studied in relative isolation. But even then the complexity of a single module can be daunting. As our knowledge increases, diagrams of gene regulatory networks look increasingly like explosions in a spaghetti factory. We need fresh methods to explore the behaviour of such networks.

On page 188 of this issue¹, von Dassow and colleagues describe how they have taken a computational approach to the problem. Their starting point is the network of genes that makes body segments in the fruitfly *Drosophila* (Fig. 1). In the past 20 years, genetic analysis has produced a wealth of experimental data on segmentation, and a textbook model of how dynamic intercellular signalling maintains a stable boundary between cells that are in distinct states^{2,3} (see Box 1).

From these data, von Dassow *et al.* abstracted a set of key interactions which, they hoped, would constitute a discrete developmental module. To make it computationally tractable they had to simplify it further. Their final model has 136 coupled equations with nearly 50 free parameters for such values as half-lives, diffusion constants and binding coefficients of the gene products involved. For virtually none of these are real values known. So the authors had to use random sampling and statistical methods to explore the properties of this model over the plausible range of parameter values. The boxed item in the paper (page 189) shows the proteins and type of equations involved.

The prime result is counterintuitive: a random search of parameter space identified a surprisingly high frequency of 'solutions' that allowed the model to generate the correct pattern of segment gene expression; correct, that is, in the sense of resembling the pattern actually observed in developing *Drosophila* embryos. The values of parameters in these solutions do not converge on a single set of optimal values — the value of almost every parameter can range over orders of magnitude and still be compatible with a correct solution. It is the organization of the gene network that provides stability, not the fine-tuning of molecular interactions.



Figure 1 Segmentation (in this case abnormal) in the *Drosophila* embryo. Normally the segment-polarity gene network generates a linear array of 14 segments, each bounded by a stripe of cells expressing the *engrailed* gene. This 'two-tailed' embryo shows how the segment-polarity network can make regular patterns, even with abnormal inputs. Signals from the mutant mother have triggered two arrays of segments to form back-to-back. Where they meet, a circular segment boundary has formed (here revealed by staining for Engrailed protein).

It is revealing, though, that when the group first built the model it did not work at all. Despite their best efforts to distil a logically complete model from data in the literature, and from discussions with experimental workers, no parameter set provided a correct solution. Certain essential linkages in the network were missing. In a confession of humility that is all too rare among scientists,

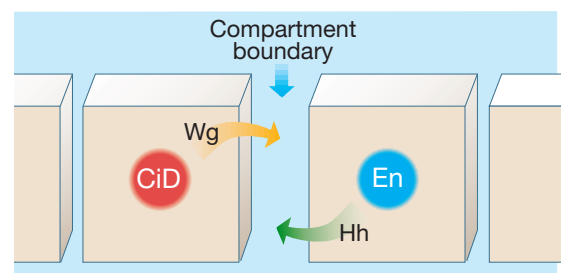
the authors point out that "Biologists' maps of gene networks are rapidly outgrowing our ability to comprehend genetic mechanisms using human intuition alone, as shown by our initial failure".

With hindsight, logical gaps in the textbook model are not difficult to spot, but no textbook or review had highlighted them. For example, it was not clear why the *engrailed* gene is not activated in front of, as well as behind, the cells expressing *wingless* (Box 1). Von Dassow and colleagues have plugged this and other gaps with plausible assumptions based on hints already buried in the experimental literature; in this case, that the gene transcription factor CiD represses *engrailed* transcription in the absence of signalling from another protein called Hedgehog. This highlights a key aspect of the work: the model is so closely tied to real data that its inadequacies immediately define a programme of experimental work to test these assumptions.

With the segmentation gene network implemented *in silico*, it becomes possible to explore how different sorts of variation affect its output. The effects of varying parameter values over small ranges are equivalent to

Box 1 Making segments in *Drosophila*

Cells on either side of the early segmental boundaries are maintained in distinct states by an exchange of signals between them. Anterior cells are defined by expression of the gene transcription factor *Cubitus interruptus* (CiD), and secrete a signalling protein called *Wingless* (Wg). Posterior cells are defined by expression of the *Engrailed* transcription factor (En), and secrete the *Hedgehog* signalling protein (Hh). Von Dassow *et al.*¹ have modelled the interaction between these two intercellular signalling pathways, and the transcriptional control of the relevant genes. This forms the heart of the 'segment-polarity' gene network. Once triggered, it is self-sustaining².



In *Drosophila*, the segment-polarity network is initially cued by a purely transcriptional patterning system that acts while the embryo is still a single multinucleate cell. This system is mediated by the 'pair-rule' segmentation genes, so called because they generate a pattern of double segment periodicity. Different combinations of pair-rule gene products activate *engrailed* and other segment-

polarity genes in a periodic pattern, thereby defining the length of each segment repeat.

The segment-polarity network probably works in much the same way in all insects, and perhaps more widely. But the inputs that trigger it may be different in insects, such as grasshoppers, in which patterning of the segments occurs after cells have formed⁴⁻⁶. **P.D. & M.A.**

those of minor mutations; small changes in the inputs used to trigger patterning mimic the natural variation in development seen from embryo to embryo. On both of these counts, the network is satisfactorily robust. Most small changes in parameter values have little effect — only at rare thresholds does the behaviour of the model switch from one stable state to another.

Initial conditions can also be varied more widely, to explore how this gene network might behave in different developmental contexts. For example, the model was designed with a precise periodic input to trigger activity of the segment-polarity genes throughout the whole length of the body. This mimics what happens in *Drosophila* (Box 1). In many other insects, however, segmentation spreads through a field of cells from head to tail (much as it does during early patterning in vertebrate embryos). In these cases, the segment-polarity system seems to be conserved⁴, but the upstream triggers may not be^{5,6}. Von Dassow *et al.* are quite happy with this: their model will generate the same segment pattern with a variety of different inputs, and the inputs can be much less precise than those known from *Drosophila*.

Our understanding of gene networks is at an early stage. We perceive their complexity only after it has been filtered by the limitations of the techniques used to study them. Genome databases and DNA-chip technology, which enables huge numbers of genes to be screened for activity, will undoubtedly provide more, and much more complicated, data than anything produced by *Drosophila* genetics. If a relatively simple gene network such as the segment-polarity system is hard to understand intuitively, we can be certain that modelling will be essential to make sense of the flood of new data.

But this will not be elegant theoretical modelling; rather, it will be rooted in the arbitrary complexity of evolved organisms. The task will require a breed of biologist-mathematician as familiar with handling differential equations as with the limitations of messy experimental data. There will be plenty of vacancies, and, on present showing, not many qualified applicants.

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Laser physics

The smallest random laser

Diederik Wiersma

How small can you make a light source? A common light bulb is a few centimetres in diameter. You can make light bulbs several millimetres across, but for a light-emitting device much smaller than this, we should turn to lasers. Lasers are nowadays widely used in industry, in hospitals and in many devices that we have at home. A compact disc player, for instance, makes use of a miniature laser diode only a few millimetres big. In two papers in *Applied Physics Letters*¹ and *Physical Review Letters*², Cao *et al.* describe a laser that is a thousand times smaller still: their laser is a small grain, 1.7 micrometres in diameter — about one-tenth of the diameter of a human hair.

The microlaser created by Cao *et al.* is not the smallest ever laser source, but it is a special type of microlaser. It uses a highly disordered structure to obtain laser action. The behaviour of light waves in disordered structures is highly complex, yet disordered materials are familiar to us all. Every substance that looks white falls into this category, including paper, white paint, fog, marble and a glass of milk. The study of the behaviour of light in such disordered materials is an active field of research^{3,4}.

A light wave that passes through a white object like a glass of milk will undergo a process called multiple scattering. Milk is a suspension of many small fat droplets, each with a strong tendency to scatter light: when a light wave hits a fat droplet its direction of travel will be changed in an arbitrary way. A light wave passing through a glass of milk will be scattered thousands of times by

thousands of fat droplets. This is what gives milk and all other disordered substances their opaque white appearance.

It is this mechanism of multiple scattering that Cao *et al.* use to make a tiny random laser. However, multiple scattering alone is not enough to make a laser. A laser requires two ingredients: a material that amplifies light, and some feedback mechanism that (temporarily) traps the light in order for the amplification to be efficient. In normal lasers the trapping element is a cavity — two mirrors facing each other with the amplifying material in between. The light passes back and forth between the mirrors, thereby passing several times through the amplifier, until it leaves through one of the mirrors that is partially transmitting (Fig. 1a).

In the case of a random laser the cavity is replaced by multiple scattering. In 1967, Letokhov⁵ predicted that the combination of multiple scattering and light amplification would lead to a form of laser action. Nonetheless, it was 25 years before random laser action was observed experimentally⁶. It became clear that the multiple scattering of light that takes place in a disordered material does not really provide a feedback mechanism, but it makes the light stay inside the material long enough for the amplification to become efficient. Instead of bouncing from one mirror to another, the light waves bounce from one particle to another thousands of times before they leave the disordered material (Fig. 1b). Because the multiple scattering is completely random, the term 'random laser' is used. The emission charac-

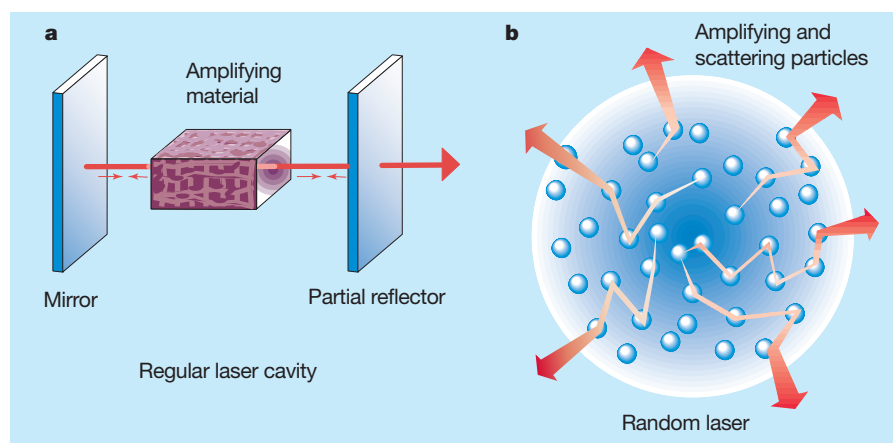


Figure 1 Comparison between a regular laser and a 'random laser'. **a**, In a regular laser the light bounces back and forth between two mirrors that form a cavity. After several passes through the amplifying material in the cavity, the gain amplification can be large enough to produce laser light. **b**, In a random laser the cavity is absent but multiple scattering between particles in the disordered material keeps the light trapped long enough for the amplification to become efficient, and for laser light to emerge in random directions.

teristics of a random laser are similar to those of a normal laser: the emission spectrum can be extremely narrow, which means that the colour of the emission is well defined, and the output can be pulsed⁷⁻⁹. But unlike a regular laser, a random laser will emit randomly in all directions, just like the emission from a common light bulb.

The tiny random laser built by Cao *et al.*^{1,2} consists of disordered clusters of zinc oxide (ZnO) nanocrystals. After excitation by an external light source, these ZnO nanocrystals provide both the amplification and the random scattering needed for random laser action. They are also easy to make and extremely cheap: one ZnO cluster costs much less than 1 cent. In addition, the laser characteristics can be easily tuned by varying the geometry of the clusters. Each cluster will operate at its own specific wavelength, depending on its shape and size.

One might question how laser action occurs in a disordered material, given that it lacks a real cavity (Fig. 2). The answer is simple. The condition for lasing comes from a careful balance between gain and loss. The gain depends on how much time the light spends inside the amplifying material; the loss depends on how easily the light can escape. Lasing simply occurs when the gain becomes larger than the loss. For example, within a sphere of disordered amplifying material of radius a , the gain is proportional to its volume ($4\pi a^3/3$) and the loss is proportional to its surface area ($4\pi a^2$). This means that, upon increasing the volume, it is possible to reach a situation where the gain becomes larger than loss and the system starts to lase. The threshold volume does not necessarily have to be very big: in the case of random ZnO clusters it is only a few cubic micrometres.

Although random laser action in ZnO clusters can be explained by multiple scattering, the details are still vague. Apart from the trapping of light by multiple scattering, other processes can play a role. If the scattering is very strong, light waves can start to bounce randomly in closed loops and get trapped. This is the mechanism behind localization of light in a disordered medium^{3,4}, a peculiar phenomenon in which light transport comes to a complete stop. But the conditions for obtaining localization are very strict, and not likely to exist in ZnO clusters. More common is additional feedback from the surface of the sample. The outer surface of a cluster will reflect light back inside, which enhances the entrapment. Especially for micro-size systems, such as small clusters or films, feedback from the boundaries is expected to be an important factor. More theoretical and experimental work is needed to understand these random microlasers in detail.

Reducing the size of a laser source to a few micrometres opens up many possibilities. It



Figure 2 Model of an amplifying disordered material used to build a 'random laser'. Green light is multiply scattered by the spheres and charges the system, thereby enabling the amplification process. The red light is both multiply scattered and amplified. The combination of multiple scattering and amplification leads to random laser action. Miniature random lasers can be used as sources in optical devices, such as wave-guides or all-optical switches. Their tiny size also makes them suitable for marking documents or materials in a hidden way.

allows, for instance, the integration of a laser within tiny optical devices. There is a growing effort to develop materials, called photonic crystals, that can guide and switch light waves in the way that electronic devices control electric currents. A random microlaser would play the crucial role of the active element or miniature light source in such crystals. But this is just one of many possible applications. On a different note, random microlasers can be used to monitor the flow of liquids by adding a small amount of ZnO clusters to the liquid and detecting the laser emission over large flow distances. Furthermore, its specific wavelength of operation, depending on shape and size, makes the miniature random laser suitable for encoded marking of documents or materials. The presence of a specific ZnO cluster could be detected by monitoring its particular laser emission, but would be invisible to the human eye. ■

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100 YEARS AGO

Mr. E. G. Green, Government entomologist at the Botanic Gardens at Peradeniya, Ceylon, has recently been able to confirm by personal observation the web-spinning habits of the red ant (*Ecophila smaragdina*). He has seen ants actually holding larvæ in their mouths and utilising them as spinning machines. To find what would be done, some leaves which had been newly fastened together by ants were purposely separated by Mr. Green. The edges of the leaves were quickly drawn together by the ants, and, about an hour later, small white grubs were seen being passed backwards and forwards across the gaps made in the walls of the shelter. Each grub... was held in the jaws of one of the worker ants, and its movements directed as required. A continuous thread of silk proceeded from the mouth of the larva, and was used to repair the damage. There were no larvæ amongst the occupants of the disturbed inclosures, and the grubs used for spinning were apparently obtained from a nest a short distance away, which probably accounts for the considerable time that elapsed before the rent was repaired. From *Nature* 12 July 1900.

50 YEARS AGO

The Royal Canadian Air Force, Engineering Division, has carried out investigations upon aircraft de-icing for some time past, and now considers that thermal de-icing, or actually anti-icing, appears to hold more promise than either the heated surface, mechanical pulsation, or chemical treatment hitherto employed. It has equipped a large four-engined Rolls-Royce Merlin-powered 'North Star' aircraft with the necessary apparatus for flying tests and observation, and intends to collect meteorological data upon cloud conditions... as well as to experiment upon the dispersal or prevention of ice accretions. The principal feature of the 'Ice Wagon' is a large 'shark's fin' on the top of the body. This will be fitted with the electro-thermal de-icing devices, and has blister-type observation domes on either side from which an operator can study and control the ice-shedding process during flight. The propellers are also fitted with similar electric blade heating... The general principle of the new technique is one of intermittent flow of current along wires installed at places where the ice that is forming is most readily dislodged. This is considered to be more efficient than continuous heating of a surface. From *Nature* 15 July 1950.

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Apoptosis

The little devil of death

Vincenzo De Laurenzi and Gerry Melino

Cell suicide is an essential, everyday event in developing and adult multicellular organisms. Indeed, alterations in the ratio of proliferating to dying cells result in several diseases, including cancer and autoimmunity. But cells must not die without good reason, and ‘roadblocks’ at several points in the cell-death signalling pathway prevent wanton cell suicide. These blocks must then be shifted when necessary. Writing in *Cell*, Verhagen *et al.*¹ and Du *et al.*² identify a new protein — alternatively called Diablo¹ or Smac² — that unblocks one step in the cell-death pathway. Their work reveals another way in which cells fine-tune their response to death signals, and provides a new potential target for therapeutic intervention.

Apoptosis — the process of programmed cell suicide — relies on a cascade of enzymes. Most of these are caspases, enzymes that are expressed as inactive precursors (procaspases) and are activated by being cleaved at specific peptide bonds. Likewise, activated caspases in turn activate procaspases at the next point in the enzyme chain by cleaving them at specific bonds. Caspases early in the cascade are activated after the binding of an extracellular death signal to its receptor on the cell surface. A little later in the cascade, caspase activation relies on the release of a protein (cytochrome *c*) from mitochondria (Fig. 1).

Activation of all of these caspases requires that they be recruited into large, multiprotein ‘death complexes’, called the death-inducing signalling complex for caspases early in the chain, or the apoptosome for the caspases activated later on. Large domains in the caspases may allow them to bind to molecules that link to the multiprotein complexes³. Caspases that act even later in the process lack these interaction domains and cannot be recruited into the death complexes. They are activated only by other active caspases⁴.

Several different proteins act as roadblocks in the apoptosis pathway by controlling the ability of caspases to bind to linker molecules. For example, proteins of the inhibitor-of-apoptosis (IAP) family can inhibit cell death by binding to procaspases and activated caspases, thereby blocking their processing and their activity (Fig. 1)^{5,6}.

IAPs were first identified in baculovirus as proteins that suppress cell death and so allow the virus to replicate in infected cells. They were later found in other viruses, yeast, the fruitfly *Drosophila melanogaster* and vertebrates. It is not yet clear how these proteins are regulated in many organisms⁵, but, in *Drosophila*⁷, IAPs are themselves inhibited by interacting with signalling proteins called Grim, Reaper and Hid. These interactions stop the IAPs from binding to caspases, allowing the caspases to be activated and the death programme to progress⁸.

Until now, a similar mechanism had not been found in vertebrates, and no structural or functional homologues of the *Drosophila* Grim, Reaper or Hid genes had been described. But now, Verhagen *et al.*¹ and Du *et al.*² have identified and characterized Diablo/Smac as a vertebrate protein that seems to be the functional analogue of Reaper, Grim and Hid. Diablo/Smac, when transfected into human cells, renders them

sensitive to ultraviolet-light-induced cell death by binding to IAPs and removing this block in the death pathway.

However, Diablo/Smac does not share any similarity in amino-acid sequence with either its functional relatives in *Drosophila* or any other known proteins. So is this parallel function of molecules that are unrelated at the sequence and structural levels an example of convergent evolution? Might there be other vertebrate proteins that share both function and structure with *Drosophila* Grim, Reaper and Hid? This seems possible, given that, in contrast to the *Drosophila* proteins, Diablo/Smac cannot induce apoptosis directly; its effects seem to be limited to sensitizing cells to apoptosis.

Another difference between Diablo/Smac and its functional analogues in *Drosophila* is that the vertebrate protein has at its amino terminus a short amino-acid sequence that directs it into mitochondria. This ‘signal peptide’ is removed from Diablo/Smac after the protein is imported into mitochondria, but Diablo/Smac remains associated with the mitochondrial membrane and is released at the same time as cytochrome *c*. Once released into the cytoplasm, Diablo/Smac binds to IAPs, allowing caspases at the apoptosome to be activated (Fig. 1).

Several questions are raised by these results, such as how Diablo/Smac is released from mitochondria, how it interacts with IAPs, and how the kinetic and stoichiometric properties of the apoptosome are regulated. Diablo/Smac is expressed at different levels in different tissues, being particularly

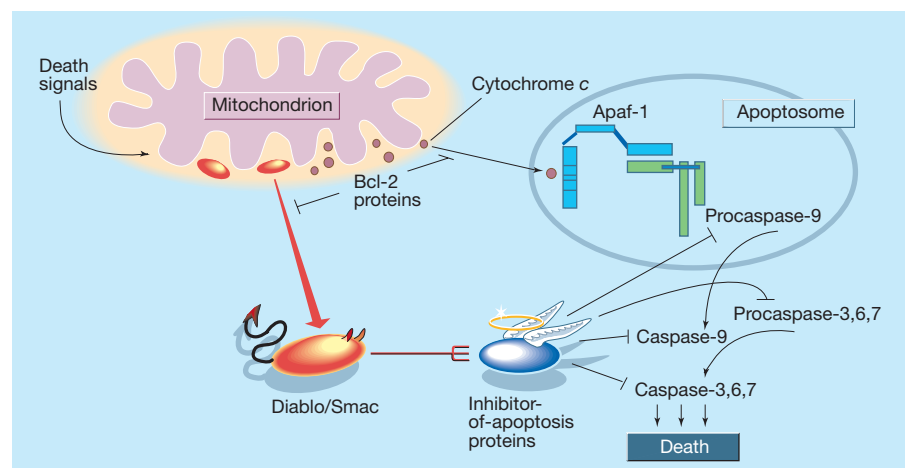


Figure 1 Fine-tuning cell death in mammalian cells. A death-inducing signal propagates to the mitochondria, which release several proteins including cytochrome *c*. Cytochrome *c*, the adaptor protein Apaf-1, the enzyme precursor procaspase-9 and dATP (not shown) form the ‘apoptosome’ death complex. Procaspase-9 is cleaved to form the active enzyme, caspase-9, which activates caspases 3, 6 and 7 in the same way. These enzymes cleave several intracellular substrates, such as ICAD (resulting eventually in DNA fragmentation) and other cell-death regulators, ensuring the death of the cell. Several different ‘roadblocks’ prevent unwanted cell death. For example, Bcl-2 proteins regulate the release of proteins from mitochondria; inhibitor-of-apoptosis proteins (IAPs) bind procaspases (to prevent them being activated) and active caspases (to inhibit their activity). Verhagen *et al.*¹ and Du *et al.*² have now shown that the IAP roadblock is lifted by Diablo/Smac, a protein that binds to IAPs and prevents them from inhibiting apoptosis. So Diablo/Smac determines cellular sensitivity to the apoptotic signal.

abundant in heart, liver and testis. Is this because of the presence of other proteins in the Diablo/Smac class in other tissues, or the existence of tissue-specific apoptosis pathways? IAPs may also act at the level of receptors for death signals⁹, so might Diablo/Smac have more regulatory targets?

These results also prompt interesting questions about disease. Viruses, such as baculovirus, may be able to extend the life of infected cells by expressing IAP proteins. Can cells in turn counteract viral infection through Diablo/Smac? Cancer cells develop by favouring proliferation over apoptosis as a result of alterations in several genes. Is loss of function of Diablo/Smac involved in the development and resistance to drugs of tumour cells? The recently identified *Drosophila* p53 protein controls apoptosis by regulating the expression of Reaper^{7,10}. Vertebrate p53 is a well-known tumour suppressor. Might vertebrate p53 also induce apoptosis by controlling the expression of Diablo/Smac or other, as yet unknown, functional analogues of Grim, Reaper and Hid?

The discovery of Diablo/Smac reveals the process of apoptosis at the level of the apoptosome to be more complex than suspected,

but with a greater capacity for regulation. Sealing death-inducing proteins away in the mitochondria offers one level of control. Then, the regulated release of these proteins needs to be accompanied by the formation of a multiprotein complex — the apoptosome — whose propensity to induce death is antagonized by IAPs. Now we have the anti-antagonist, in the form of Diablo/Smac. Yet more complexity may well exist. It seems that the cell has a greater say in its own destiny than we thought, and that scientists have yet more opportunities for manipulating cell death. ■

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This linear dependence is so well established that geochemical studies using oxygen isotopes routinely use $^{18}\text{O}/^{16}\text{O}$ variations only, ignoring the redundant variations in $^{17}\text{O}/^{16}\text{O}$.

One striking departure from this linear mass-dependence occurs for the chemistry of ozone, as has been shown both in the laboratory³ and in the stratosphere⁴. In the synthesis of ozone from oxygen, both ^{17}O and ^{18}O are enriched in the ozone by similarly large factors; the resulting ozone therefore contains an apparent excess of ^{17}O with respect to the expected mass-dependent concentration. By gas-phase exchange reactions, this ^{17}O excess can be transferred to other atmospheric molecules such as CO_2 , CO , N_2O , OH and so on⁵. Thus, various oxidizing trace-gas species in the atmosphere acquire an isotopic signature that distinguishes them from components of the rest of the Earth.

What Bao *et al.*¹ have shown is that some sulphur-bearing atmospheric compounds, specifically dimethyl sulphide from the oceans and sulphur dioxide from volcanoes, may be oxidized in the atmosphere. They therefore acquire the tell-tale excesses of ^{17}O , which can then be incorporated into sulphate minerals in solid deposits on the Earth's surface. The specific deposits the authors analysed were soils derived from sulphate-rich rocks in the Namib Desert (Fig. 1), and volcanic ash-beds in the western United States.

At this early stage, the observations of excess ^{17}O in sulphate in soil and ash deposits yield only the qualitative conclusion that an atmospheric oxidation signature has been preserved in them. Further studies, exploiting $^{18}\text{O}/^{16}\text{O}$ fractionation and sulphur-isotope effects, will be necessary to establish

Atmospheric chemistry

Rock signature from the sky

Robert N. Clayton

How can the long-term chemical evolution of the Earth's atmosphere be traced through time? Samples of 'fossil air' are available from some ice cores, but they provide evidence for only the past 400,000 years. Longer-term records of atmospheric chemistry must be sought in rocks that have acquired some recognizable chemical signature through interaction with the ancient atmosphere.

In the paper on page 176 of this issue, Bao *et al.*¹ show that such a signature can be found in some sulphate rocks, which preserve a characteristic oxygen-isotope pattern derived by atmospheric oxidation of sulphur-bearing gases. This pattern derives from atmospheric ozone; ozone is synthesized from oxygen, so the pattern may serve as a way to monitor atmospheric oxygen in the past.

Variations in the abundance of stable isotopes of light elements such as hydrogen, carbon, nitrogen and oxygen are widely used in geochemical and environmental studies. The isotopes act both as natural tracers of the provenance of the materials concerned and as indicators of the conditions under which they formed or were processed. These applications were first

introduced in 1947 by Harold C. Urey², who showed how equilibrium and kinetic chemical properties of isotopes depend linearly on differences in isotopic mass.



Figure 1 Fog over the Namib Desert. Bao *et al.*¹ show that it is likely that an anomalous oxygen-isotope signature, generated in the atmosphere from ocean-produced dimethyl sulphide, has been incorporated into the desert rocks. The result could provide the basis for a new approach to analysing the chemistry of Earth's ancient atmosphere.

these results as the basis of a new quantitative tool for studying the chemistry of Earth's past atmosphere.

Going beyond our own planet's history, Bao *et al.* suggest that an analogous process may occur on Mars. From the Viking and Pathfinder analyses of Martian soil and dust, it is known that these materials contain up to about 2.5% sulphur, a high value that suggests they are derived from volcanic gases that were deposited from the atmosphere. So oxygen and sulphur in sulphates from so-called SNC meteorites (which are of

presumed martian origin), and from future samples returned from Mars, may provide information about the chemical evolution of the martian atmosphere. ■

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Neurodegeneration

One-hit neuronal death

Nathaniel Heintz

It is a rare and fortunate individual who has not witnessed the devastating consequences of neurodegenerative illness. This experience may have come from seeing the inexorable deterioration of a family member suffering late in life from Alzheimer's or Parkinson's disease, the sudden loss of motor function in patients in the prime of life suffering from amyotrophic lateral sclerosis (also known as Lou Gehrig's disease), or the tragic consequences of any of the other diseases that wreak havoc in the nervous system. Attempts to understand the neuronal death underlying these disorders have been complicated by their varying times of onset and clinical courses. Might the loss of neurons in all of these diseases follow a common principle? Or does the neuronal death in each have its own rules and regulations? On page 195 of this issue¹, Clarke and colleagues provide experimental evidence to support a common, 'one-hit' model of cell death in neurodegenerative diseases that has implications for treatment strategies.

The genes that are mutated in neurodegenerative diseases in humans and experimental animals are being identified rapidly, and *in vitro* and *in vivo* analyses are unravelling the functions of the protein products of these genes. These studies have led to the classification of neurodegenerative diseases according to the biochemical mechanisms of the mutant gene products. Thus, the 'polyglutamine diseases' and 'protein-aggregation disorders', including Alzheimer's and Parkinson's diseases, are characterized by the accumulation of aggregates of abnormally folded peptides or proteins. In the 'axonopathies', such as amyotrophic lateral sclerosis, alterations in the morphology of neuronal axons and in the expression of genes encoding neurofilaments (part of a neuron's cytoskeleton) appear. And the 'ion channelopathies' show perturbations in the flux of ions across the neuronal membrane^{2–6}.

So there are obvious differences in the biochemical pathways that are altered in different neurodegenerative diseases. Despite this, it has been suggested that the neuronal death in all of these disorders might occur according to a common principle. One such theory that has been proposed is the 'cumulative-damage' hypothesis, which holds that the accumulation of macromolecular damage or a toxic substance over time leads to the eventual demise of the affected neuronal population (see references in ref. 1). Alternatively, cell death might result from defects in neuronal metabolic balance as a consequence of aberrant signalling⁷.

The cumulative-damage hypothesis predicts that the probability of cell death in the affected neurons will increase over time. This translates into a sigmoidal decline in cell number over time. So, early in the disease, few cells have accumulated enough of the toxic product or enough damage to trigger cell death, and few cells die. Late in disease progression, most neurons have accumulated sufficient toxic product, resulting in a large increase in the rate of cell death.

If the cumulative-damage theory does not hold, however, one would expect the probability of cell death to either remain constant or decline over time. This would translate to an exponential decline in cell number as the disease progresses. For example, if half of the cells in the affected structure die each year, then, after the first year, half of the cells would remain; after year two, a quarter would remain; and so on. To distinguish between these two possibilities for a specific disease, one need simply measure the rate of cell loss over time to determine whether it conforms to a sigmoidal or an exponential decline. No knowledge of the biochemical mechanism initiating cell loss, or of the specific cell-death pathway active in the dying cells, is required.

Clarke *et al.*¹ have used this prediction to test and exclude the cumulative-damage hypothesis for a broad spectrum of neurodegenerative conditions. These include 12 different models of photoreceptor degeneration, 'excitotoxic' cell death *in vitro*, loss of cerebellar granule cells in a mouse model, and Parkinson's and Huntington's diseases. In this range of diseases, five different neuronal types are affected. The different disorders result from mutations that cause differing biochemical alterations in the cell, and occur in both the neurons affected directly by the mutations and those dying as a consequence of the initial cell deaths. The data were collected in the authors' laboratories or taken from independent published studies of the kinetics of cell loss in specific neurodegenerative situations.

In each of these cases, the rate of cell death is best fit by an exponential decay, arguing against models in which the accumulation of damage or build-up of a toxic substance determines the rate of cell loss. Clarke *et al.*¹ propose instead that affected neurons are in an abnormal state (which they call the "mutant steady state") in which there is an increased probability that a "single rare catastrophic event" will lead to cell death. This one-hit model of cell loss may be common to many forms of neurodegeneration. This hypothesis does not require the biochemical mechanisms participating in cell loss to be defined, nor does it dictate the mechanism by which the neurons die. But a specific cell-death process called apoptosis is important in many of the diseases studied, including the retinal degeneration models, death of cerebellar granule cells in response to being deprived of the proper neuronal targets⁸, and excitotoxic cell death *in vitro* and *in vivo*⁹. Although the evidence for a role of apoptosis in Parkinson's and Huntington's diseases is less clear, the single rare catastrophic event in all of these diseases could well be the irreversible commitment of single cells to the apoptotic death cascade⁷.

This elegant study¹ also has implications for therapeutic intervention. As discussed by the authors, the fact that the probability of cell death in the affected population is either constant or decreases during disease progression means that the chance that a neuron can be rescued from death by pharmacological intervention does not decrease with age. However, in some well-characterized animal models of neurological disease, obvious histological abnormalities appear and deterioration of neuronal function occurs well in advance of cell loss¹. It would be interesting to find out whether the one-hit hypothesis is relevant in these models, despite the discordance between the progression of symptoms and neuronal death. Nonetheless, these results are certain to stimulate much debate and experimentation, aimed both at identifying common mechanisms of neurodegen-

eration and at developing common ways of treating these tragic diseases. ■

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Pulsar astronomy

Older than they look

John H. Seiradakis

Men who don't know the age of their girlfriends can make a reasonable guess by finding out the age of her parents. This is what Gaensler and Frail¹ have done in order to get a lower limit for the age of a pulsar observed to be fleeing away from its parent supernova. Writing on page 158 of this issue, they report a fairly firm minimum age for the supernova remnant G5.4–1.2; it turns out to be much higher than the 'characteristic age' of the related pulsar B1757–24. Given that the pulsar and supernova remnant were formed at the same moment in time, this result raises serious questions about the assumed ages of pulsars.

Pulsars are super-dense, highly magnetized, rapidly rotating neutron stars that are revealed to us through their periodic radio emission (Fig. 1). A neutron star has a mass similar to that of the Sun (about 10^{30} kg), but is only 10 km or so across — much less than the Sun's diameter, which is about 700,000 km. Pulsars are thought to be formed in supernova explosions, when an old, giant star collapses in a huge outburst of energy and mass, leaving behind a rotating neutron star. The material blown outwards by the explosion forms an expanding supernova remnant. Pulsar B1757–24 is unusual in that it has travelled so far from its birthplace (the centre of the remnant) that it has blasted its way out of the supernova envelope (Fig. 2).

The characteristic age of pulsars is often confused with their true age. The total energy emitted by a pulsar is a combination of the radiation it gives off and an outflow of particles². These particles transfer part of their energy to the cloud of atoms surrounding the pulsar, forcing it to glow as a bright region of ionized gas (Fig. 2). As pulsars lose energy, through their emitted radiation, their period of rotation, P , becomes longer. Clearly, their present period of rotation depends on both the initial period and the rate of its increase ($\dot{P}$). It turns out that, by assuming a very fast initial period and emission from pure radiation (not including particles), it takes $\tau = (P/\dot{P})/2$ years for a pulsar to reach a period P . This should be close to the true age of the pulsar and is usually referred to as its

characteristic age. Earlier measurements³ gave a characteristic age of 16,000 years for pulsar B1757–24.

Gaensler and Frail¹ use two high-resolution radio maps of the supernova remnant G5.4–1.2, taken six years apart (1993 and 1999), to calculate the rate of its expansion. They find a rather slow rate, leading them to estimate a minimum age of 39,000 years for the supernova remnant. Given the characteristic age of the pulsar and the distance it has travelled from the centre of the supernova remnant, this means the pulsar has to be moving very fast ($1,500 \text{ km s}^{-1}$). This velocity is extremely high and cannot be explained by models of supernova explosions. The simplest explanation is that the actual age of the pulsar is the same as the age of the supernova remnant, which gives the

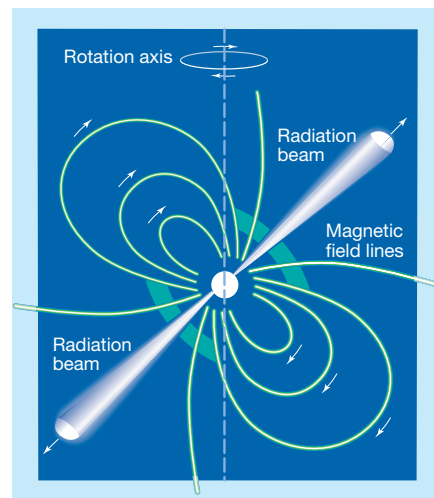


Figure 1 Older but still spinning. The pulsar's magnetic field forms a magnetic dipole (like a bar magnet) with radio waves emitted from the magnetic poles. The magnetic poles are rarely aligned with the rotation axis so, like in a lighthouse, the radio waves appear as periodic pulses rather than a continuous beam. The gradual slowing down of the pulsar as it loses radiation energy can be used to estimate the pulsar's age. New observations of a 'young' pulsar by Gaensler and Frail¹ suggest that the true ages of young pulsars may often be underestimated.

pulsar enough time to get to the edge with a reasonable velocity. Furthermore, the authors argue that detailed calculations could raise the age of the supernova remnant to 170,000 years, in any case much higher than the characteristic age of the pulsar.

This discrepancy between the characteristic age of the pulsar and the age of the supernova remnant poses a serious problem to either the association between the pulsar and the supernova remnant or the common belief that the characteristic age of pulsars represents their true age. The morphology of the remnant and the ionized region around the pulsar suggests a fairly robust association between the remnant and the pulsar (Fig. 2). So, the characteristic age of pulsar B1757–24 is in trouble. To most neutron-star specialists, this does not come as a surprise, because the assumptions underlying the calculation of pulsar ages, corroborated by observational evidence, seem to point to characteristic ages that are smaller than true ages^{4–6}. If pulsars are generally older than they appear then there must be additional mechanisms of energy loss — a pivotal part is probably played by the outflow of particles.

The age discrepancy singled out by Gaensler and Frail will probably be solved when the pulsar velocity is measured directly by estimating the position of the neutron star with great accuracy over long time

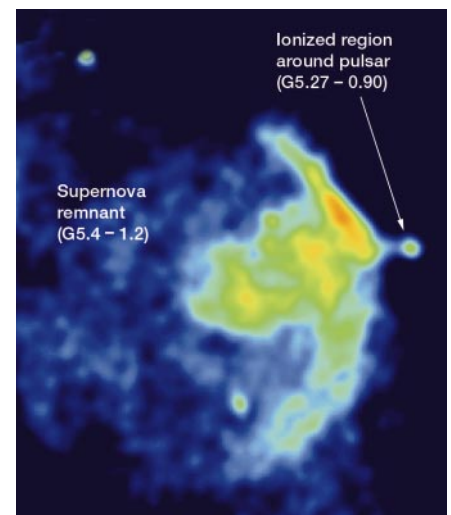


Figure 2 Pulsar bursting out of the supernova remnant like a bullet from a gun. During the massive explosion of a giant star, a few decades ago, a neutron star was born. It received a powerful kick during birth, probably due to an asymmetry in the magnetic field of the dying star, and began travelling away from the centre of the supernova remnant (G5.4–1.2). The neutron star, observed today as a radio pulsar (B1757–24), emits copious high-energy particles that ionize a region surrounding it (G5.27–0.90). The pulsar itself is so tiny that it cannot be seen even with the largest telescopes, but its tell-tale ionization reveals that it has escaped from the supernova remnant.

intervals. This can be achieved either by high-resolution mapping or by accurate measurements of the pulsar's period. To an observer on Earth, the pulsar's period becomes shorter as we travel towards the pulsar or longer as we move away. By studying these changes in the period, the pulsar position can be estimated with very high precision. This method is referred to as 'pulsar timing'. Accurate measurements of the pulsar's position will allow us to work out the pulsar's age from the distance it has travelled since its birth. We expect such observations to be completed within the next few years. The finding that young pulsars are generally

older than was thought may explain why many apparently young pulsars are not associated with a supernova remnant. ■

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Plant pathology

The bugs from Brazil

Michael Bevan

Agricultural crops are under constant threat from disease and the vagaries of climate, and in many areas of the world much human suffering stems from crop failure. Couple these factors with the demands of expected increases in human population, the continued encroachment on virgin and marginal land for agriculture, and decreasing water quality, and it is plain that food producers have a considerable challenge on their hands.

Genome sequencing is a potent new tool in the biologist's armoury, and it is being applied to a wide variety of organisms that directly or indirectly affect food supplies. A striking and welcome example of the approach comes on page 151 of this issue¹, where a Brazilian sequencing consortium report the first public sequence of a free-living plant pathogen. The organism con-

cerned is the bacterium *Xylella fastidiosa* (Fig. 1), the cause of citrus variegated chlorosis, a serious disease of citrus fruit. Certain strains of *X. fastidiosa* also affect other commercially important produce such as coffee, nuts and other fruits².

The *X. fastidiosa* genome sequence is one of 24 complete bacterial genomes now available³. Each reveals a gene complement and organization that reflects in marvellous detail the specific adaptations and lifestyles of the organism concerned.

The *X. fastidiosa* bacterium is highly specialized. It multiplies in the foregut of sharp-shooter leafhoppers, which feed on sap in a plant's xylem, the main water-conducting tissue. The insect carrier delivers bacteria directly into the xylem system of host plants. There they multiply and cause symptoms of chlorosis — chlorophyll loss and yellowing

— and the premature production of fruit which are small, tough and therefore worthless. The disease is potentially devastating; the most effective control is to produce healthy bacteria-free material for plant propagation. Brazil produces around one-third of the world's orange fruit, and nearly half of the orange-juice concentrate. So the citrus and other fresh-fruit industries are of great national significance, and research into disease control has a high priority.

The gene complement of *X. fastidiosa* reflects its life in plant xylem in three main ways. First, the bacterium is adapted to use a variety of free sugars found in xylem sap, and to supplement these sugars with glucose derived from the breakdown of cellulose, the main component of plant cell walls. Adding to the picture of a honed-down metabolism, genes encoding the enzymes needed to make sugars from amino acids and other metabolites are missing; this shows that the organism has a strict requirement for carbohydrates as the sole energy provider and source of building blocks for all biosynthetic reactions.

Second, no fewer than 67 genes are devoted to the uptake of iron and other transition metals from xylem sap, and the authors suggest that depletion of these micronutrients contributes to symptoms of infection. Third, *X. fastidiosa* produces two distinct cell-adhesion systems. One comprises a matrix of extracellular polysaccharides, synthesized by the appropriately named *gum* genes, that embed the bacteria in a matrix in the xylem, eventually leading to blockage of xylem flow and host water-stress. The other system is for bacterial adhesion to the gut and mouthparts of the insect vector, and is specified by 26 genes encoding the so-called fimbrial proteins that are needed for bacterial adhesion and translocation across cell surfaces.

The consortium¹ also identified genes encoding other molecules involved in cell adhesion that were previously associated only with human and animal pathogens. This is further evidence that disease-causing bacteria of humans and plants have common mechanisms of pathogenicity⁴.

One of the barriers to breeding and engineering plant resistance to *X. fastidiosa* is a comparative lack of knowledge of its interplay with its citrus hosts. Usually, host-pathogen specificity is conferred by interactions between avirulence factors encoded by the pathogen and host resistance factors. Surprisingly, however, *X. fastidiosa* lacks recognizable avirulence genes and the secretion system that injects them directly into host cells. The authors¹ suspect that the direct injection of bacteria into host cells by the insect carrier bypasses avirulence gene function, and that the host ranges of *X. fastidiosa* are defined by other molecules. Exploring this aspect of the bacterium's biology will provide new perspectives on host-pathogen interactions, with the ultimate

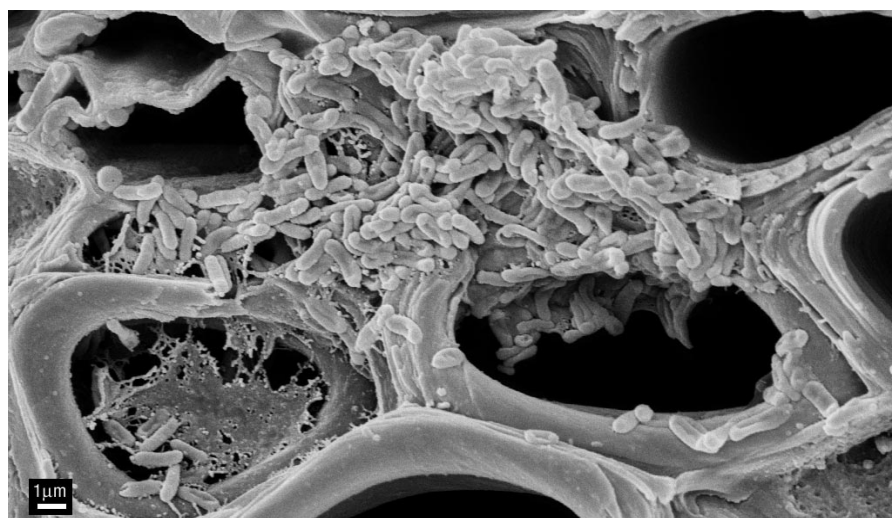


Figure 1 *Xylella fastidiosa*, a bacterium that causes severe damage to citrus trees and the citrus industry. Its genome sequence, now published¹, shows it to be highly adapted to life in a plant's water-conducting system.

general goal of developing tolerance and resistance in crop plants.

The impending completion of several other bacterial and fungal plant-pathogen sequences, and the initiation of genome sequencing of *Rhizobium meliloti* (the nitrogen-fixing symbiont of legumes), adds further to the exciting possibilities in agricultural genomics. Moreover, the near-completion of the sequence of the favoured model plant *Arabidopsis* and the start of rice genome sequencing, along with farm-animal genome projects that draw strongly on mouse and human genome sequencing, are establishing a solid foundation for research. This is research with eminently practical ends: tackling current or imminent problems in food production, human nutrition and environmental degradation. For example, disease-resistant crops, and plants better adapted to grow in extreme conditions, are nearly ready for large-scale use.

Finally, to return to South America, this first implementation of large-scale sequencing in Brazil is only a harbinger of things to come. The same consortium is now sequencing another plant pathogen, *Xanthomonas citri*. This is the cause of citrus canker, a worldwide disease that can severely damage citrus crops unless strict quarantine zones are enforced. The citrus industry in Florida is currently under threat from *X. citri* spread by a tornado in 1996 (ref. 5). The successful sequencing of *X. fastidiosa* also shows that genome projects are a highly effective tool for science policy. Such projects provide a strong direction to and framework for biological

investigations; they direct disparate areas of biology towards common themes; and they result in the distribution of the latest technologies to many laboratories, allowing scientific talent to flourish more widely. ■

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Developmental biology

Bringing two hearts together

Wolfgang Driever

Vertebrate development is a complicated business. Many structures, such as limbs, form as left–right pairs. Of other organs — such as those of the digestive system, which form by budding off the central gut tube — there is only one. And a few organs, most notably the heart, have a much more complicated genesis. The heart's origins can be traced back to the early embryo, specifically to two patches of tissue (primordia) located on either side of the vertical axis that marks the embryo's centre. During development, the heart primordia move towards this so-called dorsal midline and merge, forming the heart (Fig. 1, overleaf). How the primordia find each other has been one of life's mysteries. On page 192 of this issue¹, Kupperman and colleagues shed first light on this process, by characterizing a gene — called *miles apart* — that is essential for guiding the migration of the primordia.

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The hearts of higher vertebrates have left and right chambers, but this does not mean that the heart is initially organized bilaterally. After the heart primordia merge, a single tube forms (Fig. 1c), which then undergoes a complex looping process to generate the chambers². Because of this, the dual origin of the heart was not always clear. The first evidence came from observations of developmental defects that cause two heart tubes to form, a condition called cardia bifida. Found in a variety of organisms, from fish to humans, this disorder generally leads to death of the embryo because proper blood circulation cannot be established.

Zebrafish (*Danio rerio*) have proved a particularly useful genetic model for studying cardia bifida: their embryos are transparent, so the two heart tubes can be easily observed long before the embryos die of circulatory defects³. Systematic mutation of zebrafish genes and screening of the mutant animals has resulted in the identification of eight genes that, when mutated, cause cardia bifida³. One of these genes is *miles apart*.

In zebrafish with mutations in *miles apart*³, mesodermal tissue (from which muscle, blood and various other tissues form) forms the heart primordia in the normal way, but the primordia do not converge at the midline. Heart differentiation does not depend on this convergence — two beating heart tubes form, one on the left and one on the right of the embryo. Kupperman *et al.*¹ now show that *miles apart* encodes a receptor that binds lysosphingolipids. Members of one family of lysosphingolipid receptors transmit signals into the cells on which they are found via guanine-nucleotide-binding proteins, and the Miles apart protein belongs to this family.

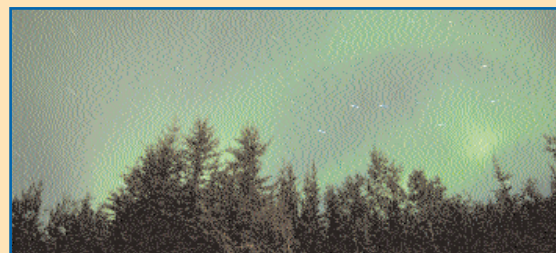
Kupperman *et al.* also show that the sphingolipid sphingosine-1-phosphate is probably the ligand that binds to the Miles apart protein to guide migration of the heart primordia. This lipid can be generated by cells from scratch or from sphingomyelin, a lipid that is stored in cell membranes⁴. Interestingly, the authors find that the Miles apart protein does not need to be expressed on the migrating heart precursor cells themselves:

Atmospheric physics

Polar lights in the Caribbean

Auroras are spectacular displays of multicoloured light (right), rarely seen at low latitudes. But users of the world's largest radio telescope in Arecibo, Puerto Rico, can now create their own light shows. As L. M. Kagan *et al.* describe in *Physical Review Letters* (**85**, 218–221; 2000), shining powerful radio waves into the night sky produces a green fluorescent glow. This provides us with the first ever pictures of clouds of metal ions in the lower ionosphere — a phenomenon that strongly disrupts radio and satellite communications.

The ionosphere is an ion-rich region of the Earth's atmosphere that reflects radio waves over long distances. The



density of ions increases with altitude in response to the ionizing effects of the Sun's radiation. A faint airglow — caused by emission of green and red light from ionized oxygen and nitrogen — can be seen at higher altitudes by sensitive detectors. But the lower ionosphere, such as the E layer, doesn't glow.

Metal ions in the E layer, torn from passing meteorites,

can build up into patches of stronger ionization that interfere with satellite signals. Intense radio waves from Arecibo were used to excite these metal ions, which in turn heat up free electrons. These energetic electrons collide with oxygen ions making them glow, as in a natural aurora. Who needs northern lights when you can have aurora equatorialis? Sarah Tomlin

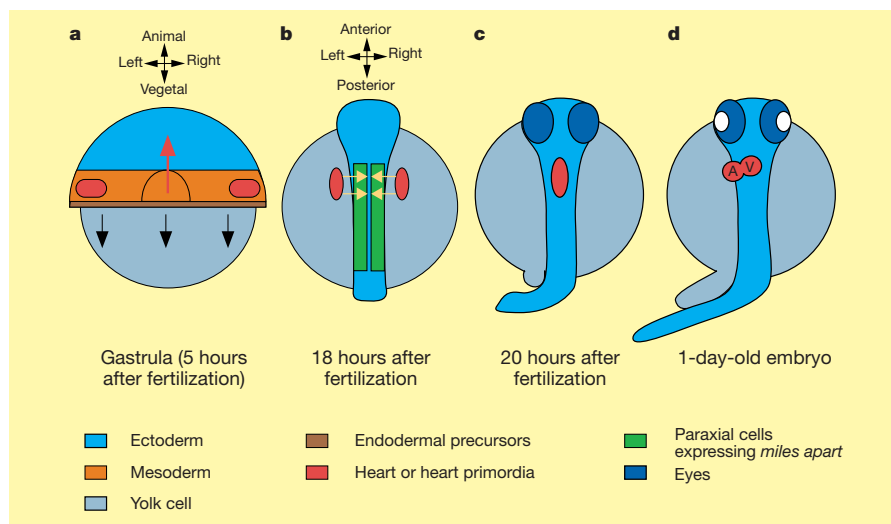


Figure 1 Making two hearts beat as one. We are looking down onto the dorsal side of the zebrafish embryo at various developmental stages. **a**, The onset of gastrulation, an early stage in development, 5 hours after fertilization of the egg. The forming dorsal axis will extend (red arrow) towards the 'animal pole' (see directional indicator), while other cells of the embryo will cover the yolk cell (black arrows). The heart precursor cells (heart primordia) form bilaterally. **b**, As shown by Kupperman *et al.*¹, stripes of cells (green) next to the midline that express *miles apart* messenger RNA are likely to be required for proper migration (yellow arrows) of the heart primordia and joining of the two bilateral groups of heart precursor cells. **c**, By 20 hours after fertilization, a single medial heart tube has formed. **d**, The heart starts to beat and circulation begins at 24 hours after fertilization; by 36 hours, looping movements have positioned the heart's atrium (A) to the left of the more centrally located ventricle (V).

such cells with mutations in *miles apart* migrate normally when transplanted into normal (non-mutant) embryos. Rather, *miles apart* messenger RNA is found in so-called paraxial cells, located either side of the midline (Fig. 1b). So the sphingosine-1-phosphate signal may tell these cells to direct cardiac precursor cells to the midline.

This is the first time that sphingosine-1-phosphate has been shown to have a role in cell migration during embryogenesis. It was already known to have a variety of effects on cell growth and survival, and to participate in cellular functions that depend on cytoskeletal filaments, including enhancing adhesion, initiating contraction and inducing protein secretion³. So several different mechanisms can be envisaged by which sphingosine-1-phosphate encourages the migration of cardiac precursor cells. For example, it could cause changes in the cell surfaces on which cardiac precursors migrate, or alter cell-cell adhesion properties. Or perhaps it encourages the release of extracellular matrix components or of a diffusible signal, which could act as attractants for cardiac precursor cells.

The *Miles apart* protein seems to be essential for the control of only a few cell-migration events during embryonic development. This is perhaps not surprising. Cell migration in the vertebrate embryo more closely resembles the tangled network of highways around Los Angeles than the tranquil lanes of a rural area. Whole sheets of cells can move, for example during gastrulation, an early stage in development. And individual cells — such as neural-crest cells, limb-muscle precursors

and many others — migrate. If we look just at the zebrafish trunk region either side of the midline, we can see heavy traffic in all directions. Heart precursors migrate towards the midline; neural-crest cells migrate away from the dorsal neural tube; germline cells move towards the posterior gonadal ridge⁵; and muscle precursors migrate from next to the midline towards the sides of the embryo to form slow muscle cells⁶.

Many distinct ligands are required to direct this flow of cellular traffic. Until now, only peptides and proteins — diffusing through the embryo or bound to cell surfaces — had been suggested to control cell migration in vertebrate embryos (see, for example, ref. 7). Lipid metabolites had been found to be involved only in the control of germ-cell migration in *Drosophila*^{8,9}. Sphingosine-1-phosphate is the first lipid metabolite known to be involved in controlling cell movement in a vertebrate embryo. The added complexity that this result is sure to bring to research on heart development only goes to prove that affairs of the heart have never been simple. ■

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Daedalus

Jet-age cleaning

Cleaning dirty surfaces is a tedious, labour-intensive task. Dirt often adheres to a surface so strongly that detergents cannot simply float it away. It has to be dislodged by mechanical scrubbing. One neat way out is the ultrasonic cleaning bath, whose detergent solution is agitated by a source of high-frequency sound. Daedalus is now extending the idea.

DREADCO engineers are fitting a standard high-pressure water-hose with a powerful piezoelectric ultrasonic generator. It launches a beam of ultrasound along the emerging water jet. Ultrasound is transmitted very efficiently by water; it can travel many tens of metres with little loss. Furthermore, the strong refractive mismatch between air and water will prevent the sound escaping from the jet and spreading wastefully out into the air. Nearly all the ultrasonic energy will hit the target, and will dislodge the dirt with great vigour. The water will then wash it away. Even if the jet breaks up into droplets during its journey, its scouring action will not be lost, for water droplets resonate strongly at ultrasonic frequencies (which is why bats don't like fog). The rapidly vibrating droplets will still deliver their ultrasonic energy to the target surface.

The DREADCO 'Ultrajet' cleaning hose will abolish many tedious jobs, notably external window-cleaning and the renovation of grimy public buildings and monuments. Scaffolding and suspended cradles, buckets and brushes and chamois leather, will no longer be needed; a simple high-velocity water-jet aimed from the ground will do the job. A thin jet of modest flow rate should be quite adequate, especially if a suitable detergent is dissolved in the water. Urban grime, peeling paint, pigeon-droppings and maybe even graffiti will be speedily and efficiently stripped from our urban eyesores. On a smaller scale, bus and train cleaning and the humble car-wash itself will be splendidly speeded and transformed.

Domestic applications are also possible. Sadly, the Ultrajet will not replace the vacuum-cleaner or the duster for internal household maintenance. But it could powerfully upgrade the action of the washing machine and the bathroom shower. An ultrasonically assisted shower would clean and exfoliate the user with a bracing ultrasonic massage. And DREADCO's Ultrajet toothbrush will vigorously dislodge dental grime and plaque, and give an unprecedented tingling-fresh sensation into the bargain. **David Jones**

A self-extending paediatric leg implant

This device spares the need for surgical intervention by simulating natural limb growth.

Biological regeneration of the extremities can occur in some organisms (starfish, for example), but humans need recourse to a mechanical solution. Growing children present a particular challenge, and those who lose bone tissue from the knee after removal of a tumour need an implant that can not only provide stability for the femur and allow motion of the knee joint, but which also accommodates rapid growth. Here we describe a self-extending leg implant (endoprosthesis) that can closely simulate natural growth and which has worked successfully in paediatric patients. The energy needed for elongation of this device is provided by the patients themselves as a result of flexure of the knee joint, thereby reducing the number of operations and the risk of infection associated with manually extended implants.

The outlook for patients with malignant bone tumours has historically been poor, but now increased sophistication in medical imaging and adjuvant treatment has dramatically improved survival. The success of limb-sparing surgery has also stimulated technological developments in designing and manufacturing endoprostheses to restore function to the damaged limb.

Although bone implants have proved effective in adults^{1,2}, they cause a significant limb-length discrepancy which precludes their use in growing children. Extendible prostheses have been developed to match the growth of the opposite limb, but frequent surgery was necessary for elongation procedures, for aseptic loosening of the growing bone, and to correct failure of the extension mechanism³⁻⁷. In a long-term follow-up of uncemented endoprostheses implanted after tumour resection of the lower leg in 13 out of 44 children with growth arrest, the final clinical and radiographic results matched those of similarly treated adult patients⁸. Invasive methods involving manual, stepwise elongation of the implant also cause scar formation that limits motion of the knee joint.

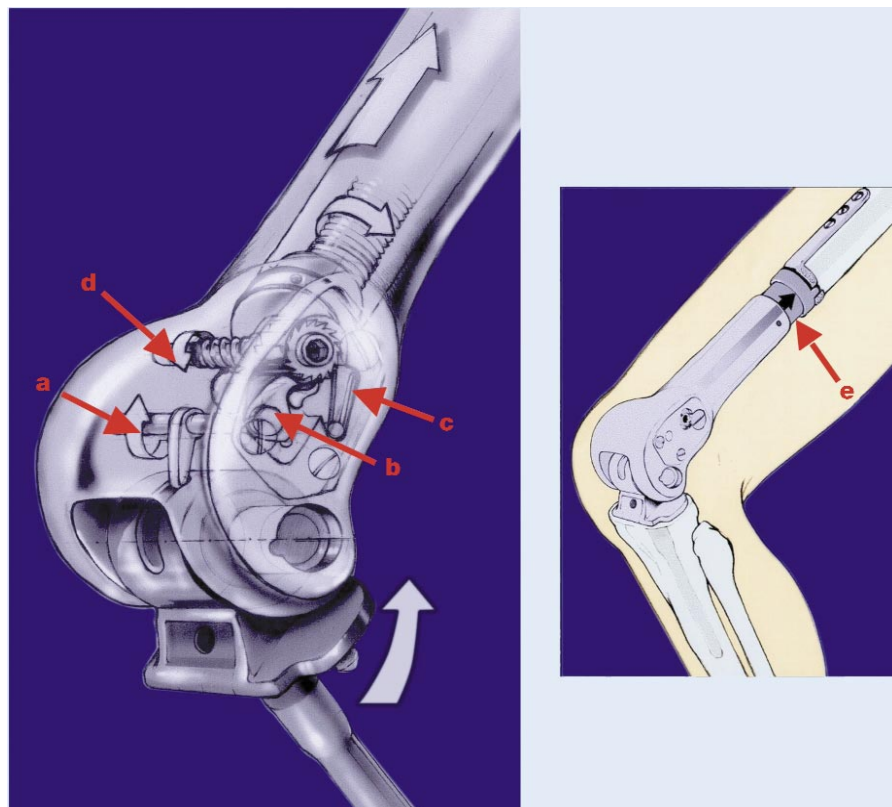


Figure 1 Function of the self-actuating extendible endoprosthesis (known as an intercondylar stepless-extension module). Flexure of the knee joint induces clockwise rotation of a switch unit (a). An angle of flexion of 100 degrees moves a gear wheel (b) via a carrier (c) by 20 degrees; this positioning is held by a spring (c). Rotation of the gear wheel is translated to a threaded spindle (d), which in turn causes a movement of 0.056 mm in a telescopic sleeve higher up the leg (e). An 18-fold repetition of this movement causes an elongation of 1 mm.

Despite their drawbacks, the application of these earlier extendible implants proved successful in 13 patients, with definitive limb salvage and equal limb length after growth was complete. These are compared in Table 1 with two patients treated with the endoprosthesis, in which continuous automatic elongation is brought about by enforced knee bending⁹.

Overlengthening of the self-extendible prosthesis is prevented by the tension of the surrounding soft tissues, which increases after each elongation procedure (0.056 mm) and is gradually decreased as the soft

tissue grows, thereby allowing the knee more flexure and stimulating the elongation mechanism. The number of surgical procedures needed for the flexion-controlled device has been reduced (Table 1). A further improvement for children under treatment now is the introduction of a central camshaft into the implant (Fig. 1), which allows the knee to bend painlessly beyond the point of elongation (100-degree bend) up to an angle of 140 degrees.

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Table 1 Comparative performance of manually and self-actuating extendible endoprostheses

	Manual device	Self-actuating device
Age at resection of tumour (yr)	11.4 (7.3–16.1)	9.3 (6.5–12.1)
Age at implantation of module (yr)	12.8 (10–18)	12.9 (10.3–15.5)
Deficit in leg length at implantation (mm)	–12 (0–40)	–48 (–45––51)
Total lengthening after implantation (mm)	84.8 (11–195)	157 (145–169)
Step lengthening (mm)	50–100 mm, by surgery	0.138 mm d ^{–1}
Number of operations*	10 (5–25)	7.5 (6–9)
Follow-up (months)	112.6 (70.6–158.1)	105.2 (100.7–109.7)

Fifteen cases (12 treated for osteosarcoma, 3 for primitive neuroectodermal tumour/Ewing's tumours) are shown who have suffered growth arrest after implantation of either a manual (n=13) or a self-actuating (n=2) growing module. Mean values (and ranges) are shown.

*Includes surgical procedures for implantation complications.

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Insect metabolism

Preventing cyanide release from leaves

Organisms that produce hydrogen cyanide gas to protect themselves against predators can do so by the enzymatic breakdown of a class of compounds known as cyanogens (such as cyanogenic glycosides)^{1,2}. Here we show how a neotropical butterfly, *Heliconius sara*, can avoid the harmful effects of the cyanogenic leaves of *Passiflora auriculata* (passion vine), on which its larvae feed exclusively. To our knowledge this is the first example of an insect that is able to metabolize cyanogens and thereby prevent the release of cyanide. The mechanistic details of this pathway might suggest new ways to make cyanogenic crops more useful as a food source.

Many organisms, including humans, possess enzymes for detoxifying hydrogen cyanide (HCN). Barriers against herbivory of cyanogenic plants may be more effective as a result of the slow or inefficient detoxification by the herbivore of HCN or of byproducts of the enzymatic breakdown of cyanogenic glycoside, which generate not only HCN but also toxic aglycones upon ingestion¹. Nonetheless, cyanogenesis is no

defence against specialist herbivores such as the larvae of *Heliconius* butterflies. We investigated how *Heliconius* butterfly larvae process cyanogenic glycosides found in their *Passiflora* host, a diverse neotropical genus in which coevolution with *Heliconius* accounts for morphological innovation in some species³.

We examined the fate of five classes of *Passiflora* cyanogens ingested by *Heliconius* larvae by surveying the distribution of cyanogens found in 12 species of *Passifloraceae* and 14 species of teneral adult *Heliconius* reared on specific hosts. We extracted cyanogens from fresh *Passifloraceae* and butterflies and identified them using published procedures^{1,4,5}.

All butterflies contained aliphatic cyanogens, irrespective of their presence in host plants, extending previous findings that these cyanogens are synthesized *de novo* by *Heliconius*⁶. Most cyanogen classes in hosts were not retained in the butterflies, nor excreted in larval frass. However, we found monoglycoside cyclopentenyl cyanogens in all butterflies fed on *Passiflora* containing these compounds. *Acraea horta*, a distant relative of *Heliconius*, is known to sequester a cyclopentenyl cyanogen from *Kiggelaria* plants⁷, but we have now demonstrated cyanogen sequestration in *Heliconius*.

Paper chromatography and high-pressure liquid chromatography of cyanogens from *H. sara* and its host plant *P. auriculata* were followed by specific enzyme tests, ¹H- and ¹³C-NMR analyses, and mass spectroscopy. We compared spectral data from these cyanogens and their tetramethylsilane–ether derivatives with published spectra for cyanogens of *Passifloraceae* and related families^{1,4,5}. The primary cyanogens in *P. auriculata* were monoglycoside cyclopentenyl cyanogen, epivolkenin (90%), its diastereomer taraktophyllin (5%) and a diglycoside cyclopentenyl cyanogen (5%). Of these, only epivolkenin was sequestered by *H. sara* (Fig. 1a).

We isolated a previously unknown metabolic derivative of epivolkenin, sarauriculatin, from *H. sara*, which we identified as (1S, 4R)-1-(β-D-glucopyranosyloxy)-4-hydroxy-2-cyclopentene-1-thiol (Fig. 1b). Sarauriculatin was present at a ratio of 1:2 with epivolkenin. This new compound was characterized as a cyclopentenyl cyanogenic glycoside that has undergone replacement of the nitrile group by a thiol, a metabolic reaction that pre-empts the enzymatic release of cyanide.

Conversion of nitrile groups is due to specific enzymatic action^{8,9}, and conversion of cyclopentenyl cyanogenic glycoside nitriles to their amides occurs spontaneously in plant isolates, probably as a result of mixing with enzymes during processing¹⁰. In repeated tests, however, we found sarauriculatin in butterfly but not in plant isolates. We conclude that a unique enzymatic mechanism exists in *H. sara* for dealing with cyanogenic glycosides.

Our results show that cyanogen sequestration in *H. sara* must be both selective and dynamic, because only one type of host cyanogen is sequestered and this is metabolized to release valuable nitrogen into the insect's primary metabolism. A fuller understanding of this process should reveal its importance in the nutrition of some species of *Heliconius*, provide a new perspective on *Heliconius*–*Passiflora* coevolution, and suggest how agriculturally important cyanogenic plants might be rendered safer and more nutritious for human and animal consumption.

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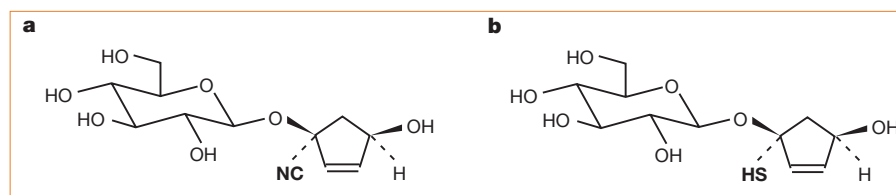


Figure 1 Structures of sequestered cyanogen from *H. sara*. **a**, Epivolkenin, and **b**, the corresponding thiol derivative sarauriculatin (epivolkenin thiol). Spectral data for epivolkenin: (1S, 4R)-1-(β-D-glucopyranosyloxy)-4-hydroxy-2-cyclopentene-1-carbonitrile ¹H NMR (250 MHz, CD₃OD): aglycone protons at δ 6.14 (dd, H-2), 6.27 (dd, H-3), 4.81 (m, H-4), 2.25 (dd, H-5A), 3.02 (dd, H-5B), ³J_{2,3} = 5.6 Hz, ⁴J_{2,4} = 1.3 Hz, ³J_{3,4} = 2.0 Hz, ²J_{5A,5B} = –14.6 Hz, ³J_{4,5A} = 4.8 Hz, ³J_{4,5B} = 7.1 Hz, β-D-glucopyranosyl protons at δ 4.62 (d, H-1'), 3.22 (dd, H-2'), 3.30–3.40 (m, H-3', H-4' and H-5'), 3.68 (dd, H-6'A), 3.86 (dd, H-6'B), ³J_{1',2'} = 7.7 Hz, ²J_{6'A,6'B} = –12.0 Hz, ³J_{5',6'A} = 5.5 Hz, ²J_{5',6'B} = 2.0 Hz. ¹³C NMR (62.9 MHz, CD₃OD): δ > 48.2 (C-5), 62.9 (C-6'), 75.0 (C-4), 82.3 (C-1), 101.2 (C-1'), 132.1 and 143.3 (C-2 and C-3), 120.6 (CN), 71.7, 75.0, 78.2 and 78.4 (remaining sugar resonances). NMR spectral data were identical for epivolkenin from *P. auriculata*. Fast atom bombardment mass spectroscopy (MS (FAB)) *m/z* 287 [M]⁺, 286 [M-1]⁺, 269 [M-H₂O]⁺, 260 [M-HCN]⁺. Optical rotation (D, 589 nm), [α]_D²⁵ + 41° (c, 0.0042 g ml⁻¹, in methanol). NMR and optical rotation data were identical for an authentic sample of epivolkenin provided by J. W. Jaroszewski. Spectral data for sarauriculatin: (1S, 4R)-1-(β-D-glucopyranosyloxy)-4-hydroxy-2-cyclopentene-1-thiol. ¹H NMR (500 MHz, CD₃OD): aglycone protons at δ 5.96 (dd, H-2), 6.09 (dd, H-3), 4.80 (m, H-4), 1.97 (dd, H-5A), 2.88 (dd, H-5B), ³J_{2,3} = 5.6 Hz, ⁴J_{2,4} = 1.3 Hz, ³J_{3,4} = 2.0 Hz, ²J_{5A,5B} = –13.9 Hz, ³J_{4,5A} = 4.6 Hz, ³J_{4,5B} = 7.3 Hz, β-D-glucopyranosyl protons at δ 4.5 (d, H-1'), 3.22 (dd, H-2'), 3.30–3.40 (m, H-3', H-4' and H-5'), 3.68 (dd, H-6'A), 3.84 (dd, H-6'B), ³J_{1',2'} = 7.7 Hz, ²J_{6'A,6'B} = –12.0 Hz, ³J_{5',6'A} = 5.5 Hz, ²J_{5',6'B} = 2.0 Hz. ¹³C NMR (500 MHz, CD₃OD): δ 45.4 (C-5), 62.5 (C-6'), 75.8 (C-4), 93.3 (C-1), 99.4 (C-1'), 134.2 and 141.3 (C-2 and C-3), 71.5, 74.8, 77.8 and 78.0 (remaining sugar resonances). MS (FAB) *m/z* 294 [M]⁺, 293 [M-1]⁺, 276 [M-H₂O]⁺. ¹H and ¹³C NMR: proton and carbon nuclear magnetic resonance, respectively; chemical shifts are given in δ (p.p.m.) and coupling constants *J* are expressed in Hertz (Hz).

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Microbiology

A triclosan-resistant bacterial enzyme

Triclosan is an antimicrobial agent that is widely used in a variety of consumer products and acts by inhibiting one of the highly conserved enzymes (enoyl-ACP reductase, or FabI) of bacterial fatty-acid biosynthesis. But several key pathogenic bacteria do not possess FabI, and here we describe a unique triclosan-resistant flavoprotein, FabK, that can also catalyse this reaction in *Streptococcus pneumoniae*. Our finding has implications for the development of FabI-specific inhibitors as anti-bacterial agents.

FabI from *Escherichia coli* catalyses the reduction of enoyl-ACP during each cycle of fatty-acid elongation^{1,2} and is inhibited by triclosan^{3–6}; InhA, the enoyl-ACP reductase from mycobacteria, is the target for the antituberculosis drug isoniazid⁷. Both drugs block the same essential step in bacterial fatty-acid synthesis. But the *fabI* gene is conspicuously absent from several bacterial genomes (Table 1) and as enoyl-ACP reduction is essential for survival, organisms lacking FabI must have a different gene to encode enoyl-ACP reductase.

We have investigated the reductase reaction in *S. pneumoniae*, in which the entire set of *fab* genes, except *fabI*, is present in a cluster spanning 10 kilobases of the genome (GenBank accession number, AF197933). A similar *fab* gene cluster was found in the clostridia. Subsets of the *fab* genes are clustered in the genomes of other bacteria, but

individual genes are also sprinkled throughout the genome¹. We found that there is one unidentified open reading frame, termed *fabK*, predicted to encode a 34K protein (FabK), within the streptococcal and clostridial *fab* clusters. The predicted protein had a centrally located FAD-binding domain⁸ (FAD is a flavin-linked redox cofactor).

We purified the FabK protein and found that it contained 0.8 molecules of FAD per FabK monomer. The protein had a specific activity of $66 \pm 4 \text{ nmol min}^{-1} \text{ mg}^{-1}$ in an *in vitro* enoyl-ACP reductase coupled-assay system, in which purified *E. coli* Fab proteins generated the *trans*-2-butenoyl-ACP substrate (Fig. 1). Another cofactor, NADH, was essential for enoyl-ACP reductase activity and FabK was able slowly to oxidize NADH, but not NADPH, in the absence of substrate. *In vitro*, FabK activity was more than 100-fold more resistant to triclosan than was FabI.

We transformed *E. coli* strain RJH13 (which harbours a temperature-sensitive allele (*fabI*(ts)) and fails to grow at 42 °C)² with a plasmid that constitutively expresses FabK and found that growth was restored at the non-permissive temperature, indicating that FabK substitutes for all of the functions of FabI.

The wild-type *E. coli* strain W3110 is sensitive to triclosan (Table 1), and over-expression of FabI and the chromosomal *fabI*[G93V] mutant could each increase resistance to triclosan 8- to 64-fold⁴. Introduction of the FabK expression vector into strain W3110 shifted the minimum inhibitory concentration (MIC) for triclosan by more than 4 orders of magnitude up to over 2,000 $\mu\text{g ml}^{-1}$. The marked resistance of these cells to triclosan confirms that FabK is not a target for triclosan and that FabI is the only triclosan target in *E. coli*.

In some bacteria, FabI is not the only target for triclosan. Organisms that contain only *fabI* are highly susceptible to triclosan (MIC < 0.5 mg ml^{-1}), whereas those carrying *fabK* are uniformly more resistant (> 2 mg ml^{-1}) (Table 1). The discovery of a triclosan-resistant enoyl-ACP reductase

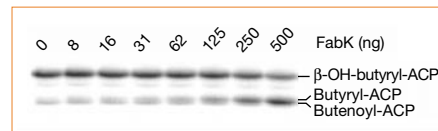


Figure 1 FabK is an enoyl-ACP reductase. Histidine-tagged FabK protein from *S. pneumoniae* was expressed in *E. coli* strain BL21-Codon Plus-(DE3)-R1L (Stratagene) and purified by metal-chelation affinity chromatography. A coupled enzyme system¹¹ was used to assay FabK by combining all the purified *E. coli* proteins required to reconstitute a cycle of fatty-acid synthesis, apart from enoyl-ACP reductase. A reaction mixture containing 100 μM ACP, 1 mM β -mercaptoethanol, 100 μM acetyl-CoA, 50 μM [2-¹⁴C]malonyl-CoA (56 mCi mmol⁻¹), 200 μM NADPH, 200 μM NADH and 12.5 $\mu\text{g ml}^{-1}$ each of FabD, FabH, FabG, FabA and FabZ in 0.1 M sodium phosphate, pH 7.0, was incubated at 37 °C for 30 min to generate the *trans*-2-enoyl-ACP substrate before being aliquoted into individual tubes, to which FabK was added to the amount indicated and incubated for 30 min at 37 °C. Products were separated by conformationally sensitive gel electrophoresis; bands were stained and their intensity quantified by using a PhosphorImager.

(FabK) in *S. pneumoniae* demonstrates that a triclosan target other than enoyl-ACP reductase is responsible for the sensitivity of this Gram-positive organism to the drug. Among Gram-negative bacteria, *Pseudomonas aeruginosa* is unique in that it not only contains both *fabI* and *fabK* in its genome but is also refractory to triclosan (Table 1). This is due in part to possession of a mechanism for active efflux of the drug⁹; however, disruption of the triclosan-sensitive *fabI* gene does not result in a growth phenotype, and enoyl-ACP activity in extracts of the *fabI*-null strain is resistant to inhibition by triclosan¹⁰. Thus, *P. aeruginosa* must contain a second, triclosan-resistant enoyl reductase, which we suggest may be a FabK homologue.

The discovery of a unique enoyl-ACP reductase has important implications for antibacterial drug development. Organisms expressing FabK are predicted to be refractory to inhibitors designed to target FabI. Conversely, selective FabK inhibitors would be effective against several important pathogens, such as the streptococci and clostridia. Organisms such as the pseudomonads and enterococci that contain both a FabI and FabK will require a combination of enoyl-ACP reductase inhibitors to block growth. Thus, antimicrobial therapy based on blocking enoyl-ACP reductase will need to be tailored for specific pathogens according to their expression of FabI and FabK.

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Table 1 Distribution and triclosan sensitivity of FabI and FabK in bacteria

Organism	Sequence identity (%) [*]		Triclosan MIC [†] ($\mu\text{g ml}^{-1}$)
	FabI	FabK	
<i>Escherichia coli</i>	100	—	0.25
<i>Staphylococcus aureus</i>	43	—	0.01
<i>Salmonella pneumoniae</i>	—	100	2.0
<i>Clostridium acetobutylicum</i>	—	58	3.0
<i>Enterococcus faecalis</i>	47	68	10
<i>Mycobacterium tuberculosis</i>	33	31	100
<i>Pseudomonas aeruginosa</i>	69	33	> 1,000

Dashes indicate that no homologous genes were detected.

^{*}Identity to FabI and FabK is shown. FabI homologues are easily recognized in both Gram-negative and Gram-positive bacterial genomes by using BLAST search algorithms, but these are not present in several pathogens. The *E. coli* FabI and *S. pneumoniae* FabK protein sequences were used to search the unfinished and complete database of microbial genomes (see <http://www.tigr.org>) for open reading frames encoding homologous proteins, and the number of identical residues was scored.

[†]Minimum inhibitory concentration (MIC) values are from ref. 12.

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Materials science

Diffusion of a polymer ‘pancake’

Thread-like chains of flexible polymers that adsorb to a solid surface assume a flat ‘pancake’ conformation¹ when the surface coverage is low and are only able to diffuse in two dimensions because so many segments are adsorbed. Here we show that the centre-of-mass diffusion coefficient of the polymer chain, measured at dilute coverage to ensure minimal chain–chain interaction, has a strong power-law dependence on the degree of polymerization. This non-linear dependence of polymer diffusion on a solid surface contrasts with the linear dependence observed on a fluid membrane².

Our system consisted of polyethylene glycol (PEG) adsorbed from aqueous solution onto a monolayer surface of self-assembled octadecyltriethoxysilane³ coated onto a fused silica coverslip to render it hydrophobic (because PEG does not adsorb from aqueous solution to hydrophilic fused silica at high pH).

Diffusion was investigated by fluctuation correlation spectroscopy^{4–6} of a mono-end-labelled fluorescent probe after two-photon excitation. The focused two-photon beam creates an illuminated spot with a beam waist of about 0.3 μm . The small number of fluorophores contained within a given volume (typically 3–10) fluctuates as polymers diffuse in and out. The fit to the autocorrelation function determines the mutual diffusion coefficient (D_M) of the fluorescing species, and $D_M \approx D$, the centre-of-mass diffusion coefficient, because the system is dilute. Experiments were followed using a Zeiss microscope with a 63 $\times$ Plan Apo-chromat objective (numerical aperture 1.4).

We varied the PEG polymer chain length (Fig. 1 legend) by a factor of 15. Polymers were allowed to adsorb for less than 5 min from dilute (1–10 nM) solution in 0.01 M aqueous phosphate buffer, pH 8.4. To study bulk solutions, PEG samples were labelled with fluorescein 5-isothiocyanate, and for monitoring surface diffusion, we used an Alexa-488 label (from Molecular Probes), a

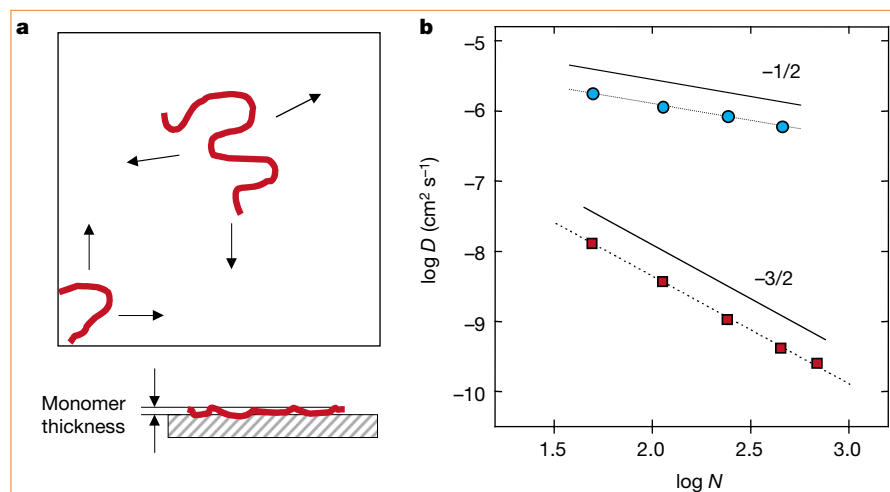


Figure 1 Comparison of polymer diffusion when adsorbed to a solid surface and in free solution. **a**, Flexible polymer chains that adsorb are nearly flat at dilute surface coverage (‘de Gennes pancake’). The sticking energy for each segment is small, so no single segment is bound tightly, but the molecular sticking energy is large. **b**, Diffusion coefficients (D) in dilute solution (circles) and at dilute coverage on a solid surface (squares) are plotted on log–log scales against degree of polymerization (N) at 22 °C, for chains of weight-average molecular weights, M_w , of 2,200, 5,000, 10,800, 20,100 and 30,500 g mol^{−1} and M_w/M_n values of 1.01–1.03 (where M_n is the number-average molecular weight).

derivatized rhodamine-green molecule. Control experiments demonstrated that unattached fluorescent labels did not adsorb, confirming that adsorption was controlled by polymer–surface attraction. At this dilute surface coverage, 0.5–2% of the saturating amount of polymer adsorbed.

We estimated the ‘sticking energy’ of the polymer as 0.5–1 $k_B T$ per segment, where k_B is Boltzmann’s constant at T the absolute temperature. This emerged consistently from analysis of the Langmuir isotherm at low surface coverage, and from Arrhenius analyses of the very slow desorption rate and of the data presented below after extrapolation to the molecular weight of a single segment. Polymer chains thus adsorb, at low coverage, in a flat ‘pancake’ conformation¹ (Fig. 1a).

Measurements of D (Fig. 1b) reveal a power-law scaling with the number of chain segments (N), so $D \approx N^{-3/2}$. This is strikingly stronger than the $D \approx N^{-1}$ relation observed for charged, semi-flexible DNA obeying excluded volume statistics but adsorbed by Coulombic attraction on a fluid lipid membrane². The key difference is that adsorption sites on a solid surface are static, so the rate-limiting events concern the polymer rather than surface. For diffusion in solution, our results are consistent with standard hydrodynamic results for chains of moderate length, when $D \approx N^{-1/2}$ (refs 7,8).

Reptation (the diffusion of a chain, snakelike, along its own length) may explain this stronger dependence of D on N for polymers under our conditions. In this model¹, the terminal relaxation time scales as $\tau_{\text{rept}} \approx N^3$. Knowing that the radius of gyration (R_g) scales as $R_g \approx N^{3/4}$ in a good solvent in two dimensions^{2,9}, and arguing

that $D \approx R_g^2/\tau_{\text{rept}}$, it follows that $D \approx N^{-3/2}$ for chains with excluded-volume statistics, as we find here. A simulation for a single self-avoiding chain diffusing among regularly spaced obstacles in two dimensions also gives $D \approx N^{-3/2}$ (ref. 10). But interpreting the value of scaling exponents is a problem, particularly as they seem to depend on obstacle density¹¹.

Reptation may be considered surprising in a dilute system where the physical origin of the static constraints that suppress lateral motion is unclear. But if there were some slack between sticking points — for example, loops of an isolated flexible chain might propagate with high probability along its length in a caterpillar-like fashion — the mathematics of the reptation model would then apply, despite the unconventional physical situation.

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Transcranial magnetic stimulation and the human brain

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Transcranial magnetic stimulation (TMS) is rapidly developing as a powerful, non-invasive tool for studying the human brain. A pulsed magnetic field creates current flow in the brain and can temporarily excite or inhibit specific areas. TMS of motor cortex can produce a muscle twitch or block movement; TMS of occipital cortex can produce visual phosphenes or scotomas. TMS can also alter the functioning of the brain beyond the time of stimulation, offering potential for therapy.

It was just two decades ago that Merton and Morton surprised neuroscientists by showing that it was possible to stimulate the motor areas of the human brain electrically through the intact scalp (transcranial electrical stimulation, TES)¹. They used a brief, high-voltage electric shock to activate the motor cortex and produce a relatively synchronous muscle response, the motor evoked potential (MEP). It was immediately clear that this would be useful for many purposes, but a problem with TES is that it is painful because of activation of pain fibres in the scalp. Five years later, Barker and colleagues showed that it was possible to stimulate both nerve and brain using external magnetic stimulation (TMS; see Box 1), with little or no pain². TMS is now commonly used in clinical neurology to study central motor conduction time (Box 2). Depending on stimulation parameters, TMS can excite or inhibit the brain, allowing functional mapping of cortical regions and creation of transient functional lesions. It is now widely used as a research tool to study aspects of human brain physiology including motor function, vision, language and the pathophysiology of brain disorders. It may even be useful for therapy, particularly in psychiatry.

Interruption of brain activity

There are several complementary methods that can be used to study the contribution of cortical networks to specific cognitive functions. Positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) have revealed the brain networks involved in specific functions. However, these methods lack time resolution and cannot alone prove that an area is essential for a particular function. TMS can transiently disrupt activity in focal brain regions, allowing researchers to assess function on a millisecond scale. TMS studies, unlike functional imaging, can also be frequently repeated.

In the visual system, TMS has been used to study perception. Whereas strong TMS of occipital cortex can produce phosphenes, a stimulus of lower intensity can induce a transient scotoma. In the initial demonstration, subjects were shown brief, randomly generated letters on a visual monitor, and TMS was delivered after the visual stimulus³. When TMS was delivered 80–100 ms after the stimulus, subjects saw a blur or nothing at all, indicating that important visual processing during that time interval had been interrupted. Subsequent studies with more sensitive techniques indicated an additional, earlier period of suppression corresponding to the initial arrival of visual information in occipital cortex⁴. In higher cortical areas, TMS can be used to tease apart specialized processing mechanisms. For example, TMS of area V5 can selectively interfere with the perception of motion of a stimulus without impairing its recognition⁵, supporting the hypothesis, originally proposed following imaging studies, that V5 is the motion perception region of the brain.

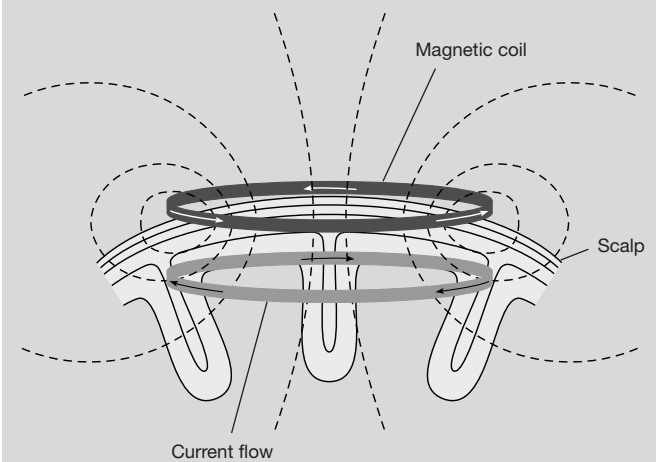
Different aspects of motor control have also been studied using TMS. In a reaction time task, a strong TMS just before the expected motor response can delay the response without altering its form⁶. High-frequency stimulation over the supplementary motor area (SMA) impairs accuracy in complex, but not simple, sequences of

Box 1

Physics and mechanism of action of TMS

For magnetic stimulation a brief, high-current pulse is produced in a coil of wire, called the magnetic coil, which is placed above the scalp. A magnetic field is produced with lines of flux passing perpendicularly to the plane of the coil (see Figure). An electric field is induced perpendicularly to the magnetic field. In a homogeneous medium, the electric field will cause current to flow in loops parallel to the plane of the coil. The loops with the strongest current will be near the circumference of the coil itself. The current loops become weak near the centre of the coil, and there is no current at the centre itself. Magnetic coils may have different shapes. Round coils are relatively powerful. Figure-eight-shaped coils are more focal, producing maximal current at the intersection of the two round components. The precise extent of neuronal activation is not known, but it clearly varies with the intensity of stimulation. TMS ordinarily does not activate corticospinal neurons directly; rather it activates them indirectly through synaptic inputs. This has been determined by the observation that TMS produces a corticospinal volley with indirect waves (I-waves) rather than with an early direct wave (D-wave)³⁹.

Single-pulse TMS, which is very safe, has been most commonly used. Devices are now available that can deliver high-frequency (1–30 Hz), repetitive TMS (rTMS). This has greater effects than single-pulse TMS, but also has the potential to cause seizures even in normal individuals. Safety guidelines have been published which should prevent problems⁴⁰.



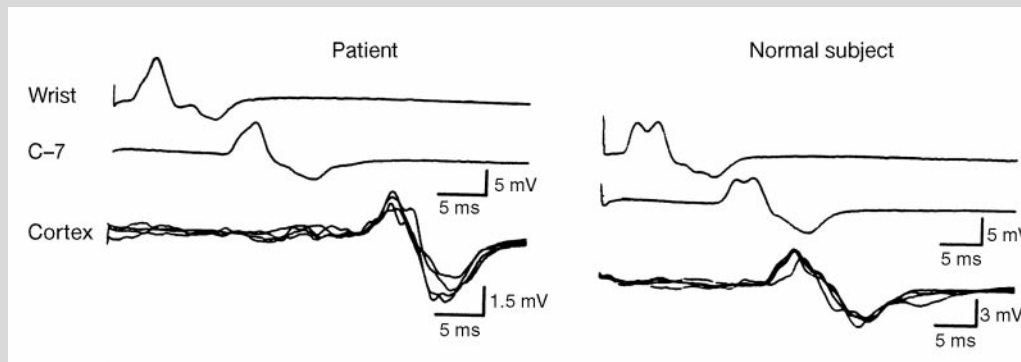
Box 2

Central motor conduction and clinical uses

With TMS, it is possible to study the speed of conduction in central motor pathways. This method has been applied in the evaluation of patients with multiple sclerosis, where there is frequent involvement of the corticospinal tract. Slowing of conduction is also commonly seen in degenerative diseases, however, making the test non-specific. Assessment of central conduction time is a useful method to evaluate neurological disturbance in patients with cervical spondylosis, where even subclinical motor disturbances can be detected⁴¹. TMS can also be used to monitor central motor tracts during spinal cord surgery.

The figure shows the results of tests of central motor conduction to the

abductor digiti minimi (ADM) muscle in a patient with extradural spinal cord compression and a normal subject. The traces show electromyogram recordings from the ADM muscle. From the top down, the responses were elicited by stimulation of the ulnar nerve at the wrist, stimulation at the C-7 level of the spinal cord (activating the nerve roots), and TMS of the cortex over the hand area, respectively. Note that the latencies of the potentials are similar in the patient and normal subject following stimulation at the wrist and spine, but the latency is much longer following cortex stimulation in the patient, implying prolonged central conduction time. With permission from Brunhölzl and Claus⁴¹.



finger movements⁷. The errors in the latter test occurred not in elements of the sequence occurring at the time of the stimulation, but in later elements. These data support a critical role for the SMA in organizing forthcoming movements in complex motor sequences.

In patients with epilepsy who are being investigated for possible temporal lobectomy, it is crucial to know which side of the brain they use for language processing. The standard test for this is the Wada test—amytal is injected into each carotid artery to suppress hemispheric function, and the side on which speech arrest is produced is determined. TMS could provide a simpler alternative test. Lateralized speech arrest can be produced using repetitive TMS (rTMS) with rates from 8 to 25 Hz for 10 s and a coil position over the left (in most people) inferior frontal region⁸. Epstein *et al.* found that speech arrest could be obtained reliably with a repetition rate of 4 Hz, an intensity of 150% motor threshold or less, and pulse trains of no more than 5-s duration over the region that gives the best motor responses for facial muscles⁹. With current techniques, however, for reasons that are unclear, the Wada and rTMS tests do not always agree.

Cortical physiology

TMS can be used to measure various parameters in motor cortex, allowing us to evaluate different aspects of cortical excitability¹⁰. Such measures are useful in understanding the changes in brain physiology seen in the setting of cortical plasticity and brain disorders. For example, the threshold for producing an MEP in resting muscle reflects the excitability of a central core of neurons, which arises from the excitability of individual neurons and their local density. As it can be influenced by drugs that affect sodium and calcium channels, threshold must indicate membrane excitability. Another measure, the recruitment curve, is the growth of MEP size as a function of stimulus intensity. This measurement is less well understood, but must involve neurons other than those in the core region that is activated at threshold. These neurons have higher threshold for activation, either because they are intrinsically less excitable or because they are further from the centre of activation by the magnetic stimulus, and would be part of the 'subliminal fringe'.

Intracortical inhibition (ICI) and facilitation (ICF) are obtained using paired-pulse studies and reflect the activity of interneurons in the cortex¹¹. In such studies, an initial conditioning stimulus is given that is large enough to activate cortical neurons but small enough that no descending influence on the spinal cord can be detected. A second test stimulus, at suprathreshold level, follows after a short time. Intracortical influences initiated by the conditioning stimulus modulate the amplitude of the MEP produced by the test stimulus. At very short intervals, less than 5 ms, the MEP is inhibited, and at intervals between 8 and 30 ms it is facilitated. ICI is probably largely mediated by neurons containing γ -aminobutyric acid (GABA). The silent period is a pause in ongoing voluntary electromyogram activity produced by TMS. Although the first part of the silent period is due in part to spinal cord refractoriness, the latter part is entirely due to cortical inhibition. ICI seen with paired pulse stimulation and the silent period reflect different aspects of cortical inhibition.

Brain plasticity

The plasticity of the mature brain—the processes involved in neural repair, and learning and memory—is one of the most exciting areas of neuroscience research today, and TMS studies have been useful in its elucidation in humans. Cohen *et al.* used TMS to study patients with traumatic, surgical or congenital amputations of the arm at about the level of the elbow¹². The areas of motor cortex targeting muscles ipsilateral and immediately proximal to the stump were larger than those for muscles contralateral to the stump (Fig. 1). These results, seen also with PET¹³, are consistent with the idea that the representation in the motor cortex of the muscles proximal to the amputation had expanded into the territory of the amputated part. An alternative interpretation is that the cortical region had become more excitable, but the expansion model is consistent with the results of animal studies using microstimulation¹⁴.

At least some of this plasticity can occur rapidly, as demonstrated in experiments in which a blood pressure cuff is used to induce reversible deafferentation. The amplitudes of MEPs produced in response to TMS in muscles immediately proximal to the temporarily anaesthetized forearm increased minutes after the onset of

anaesthesia and returned to control values after the anaesthesia subsided. On the other hand, other plastic processes may take a longer time. Mano and colleagues studied patients who had totally denervated arms after traumatic accidents and rerouting of a chest wall nerve to the biceps muscle¹⁵. They showed that projections from the biceps region of the motor cortex can be directed to the spinal cord neurons of the chest wall nerve, but this process took a year or more to occur. Expansion of the hand area was demonstrated with TMS and confirmed with PET in patients with facial palsy, showing that motor de-efferentation by itself is sufficient to trigger reorganization¹⁶.

Cortical changes also result from changes in patterns of behaviour. Indeed, it appears that there is a constant battle for the control of each neuron among its various inputs. Pascual-Leone *et al.* used focal TMS to perform detailed mapping of the motor cortical areas targeting two finger muscles, the first dorsal interosseous (FDI) and the adductor digiti minimi (ADM), bilaterally in Braille readers and blind controls¹⁷. In the controls, the motor representations of the right and left FDI and ADM were not significantly different. However, in the proficient Braille readers, the representation of the FDI in the reading hand was significantly larger than that in the nonreading hand or in either hand of the controls. Conversely, Liepert and colleagues studied cortical plasticity in patients who had a unilateral immobilization of the ankle joint without any peripheral nerve damage¹⁸. The motor cortex area of the inactivated tibialis anterior muscle diminished compared to that of the unaffected leg without changes in spinal excitability or motor threshold. In a Braille reader who took a 10-day holiday from reading, the size of the finger representation shrank dramatically until she returned to work—in fact, even time off over the weekend reduced the representation quantitatively¹⁹.

To find out how quickly such changes can occur, Pascual-Leone and colleagues looked at the motor cortical representation of the hand over a five-day period in normal subjects as they learned a skilled task with their hand²⁰. As subjects improved on a five-finger exercise on a piano, the size of the motor representation of the hand increased.

The changes resulting from proficiency in Braille reading are accompanied by other compensatory changes in blind individuals who have been deprived of vision for many years. Sadato *et al.* studied regional cerebral blood flow (rCBF) in a group of subjects blind since early infancy and a group of sighted volunteers performing the same tactile discrimination task, and found that this task activated visual primary and association areas only in the blind²¹. These results were interpreted as indicating that cortical areas normally reserved for vision may be activated by other sensory modalities in blind individuals, but the functional neuroimaging study by itself could not prove that the activation was functionally useful in the discrimination process. To address this question, Cohen *et al.* used rTMS over different scalp positions while blind and sighted subjects performed tactile identification of Braille and Roman letters²². In the blind subjects, stimulation of occipital regions induced more errors in the reading task than stimulation of any other region or stimulation in control subjects, providing firm evidence that the occipital areas are an essential component of the network involved in Braille reading in the blind. Although only a non-significant increase in errors was produced in the normal subjects with occipital stimulation, in a different somatosensory task, discrimination of grating orientation, rTMS over occipital cortex did significantly disrupt function in a normal group²³. This might reflect a more important role of visual imagery in this task.

Brain excitability in neurologic disorders

There are several different mechanisms for the genesis of epileptic seizures and for the modes of action of antiepileptic drugs. TMS can be used to give information about these mechanisms by measuring cortical excitability. For example, motor threshold is

decreased in untreated patients with idiopathic generalized epilepsy²⁴. On the other hand, in progressive myoclonic epilepsy, threshold is normal, but there is a loss of cortical inhibition demonstrated with paired pulses at 100–150 ms and an increase in facilitation at 50 ms²⁵. Specific effects can be seen with various anticonvulsants in normal subjects²⁶. Vigabatrin and gabapentin, which stimulate GABA receptors, increase intracortical inhibition; whereas carbamazepine, lamotrigine and phenytoin, which block sodium and calcium channels, elevate motor threshold. Not only can TMS elucidate these mechanisms, but it can potentially be used to quantify physiological effects in individual patients, and this may be more valuable in some circumstances than anticonvulsant blood levels.

Abnormalities of the basal ganglia can give rise to movement disorders, and this is probably in part due to their effect on motor and premotor networks in brain. Studies with TMS have revealed abnormalities in Parkinson's disease, Huntington's disease and dystonia. For example, in dystonia, there is no change in motor threshold, but there is an increase in the slope of the MEP recruitment curve and a decrease in intracortical inhibition^{27,28}. The primary dysfunction seems to be loss of cortical inhibition, and this appears to explain a number of clinical features such as activation of an excessive number of muscles in attempted voluntary movements.

Therapeutic uses

Delivery of multiple single pulses of TMS to the brain does not appear to leave any lasting effect. But rTMS can produce effects that last after the stimulation period. This was first demonstrated in the motor system. Rapid rTMS, at frequencies of 5 Hz and higher, will transiently enhance motor excitability²⁹, whereas slow rTMS, at 1 Hz, will transiently depress excitability³⁰. The mechanisms of these changes are not clear, but the analogies to long-term potentiation (LTP) and long-term depression (LTD) of individual synapses in the central nervous system are apparent.

The observation that TMS, delivered in just the right way, can speed up the reaction time in patients with Parkinson's disease led to the idea that rTMS might be useful for therapy³¹. In an initial trial using a motor coordination task, this seemed to be the case³¹, but the finding was not reproduced³². On the other hand, another study has

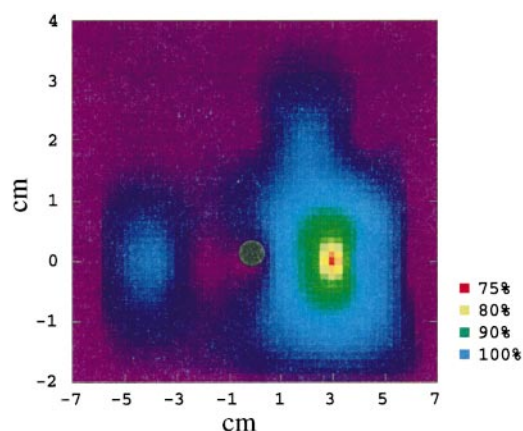


Figure 1 TMS mapping of left and right biceps from a 78-year-old subject 11 months after a left upper limb amputation. The map shows a portion of the scalp, with the top of the figure towards the front and the left side towards the left; the black dot is Cz, indicating the standard position in electroencephalography at the middle of the vertex. The map of the left biceps is over the right hemisphere, and the map of the right biceps is over the left hemisphere. Thresholds for activation at different scalp locations are indicated. Note that the muscle representation area for the left biceps is larger than that for the right biceps and that the threshold for activation of the left biceps was lower than that for the right biceps. Modified with permission from Hallett *et al.*¹².

suggested an improvement in pointing performance after rTMS³³. A different rationale was developed for why rTMS might be useful in dystonia. Physiological findings in dystonia reveal an decrease in intracortical inhibition. As rTMS delivered over the primary motor cortex at 1 Hz can increase inhibition, it was proposed that it might ameliorate the deficit. A study showed normalization of the intracortical inhibition and a modest improvement in performance³⁴.

Following the observation that rTMS might affect mood in normal individuals, a number of groups sought to determine whether rTMS might be effective in the treatment of mood disorders. Early observations have indicated that high-rate rTMS applied to the left dorsolateral prefrontal cortex may improve depression, giving hope that this might replace electroconvulsive shock treatment (ECT)^{35,36}. Conversely, low-rate rTMS delivered to the right dorsolateral prefrontal cortex may also be helpful³⁷. Therapeutic effectiveness depends on the exact site of stimulation, intensity and the precise pattern of pulses including rate, train length, intertrain interval and number of trains. This is clearly difficult to clarify and is currently a very active area of psychiatric research.

The future

Single-pulse TMS is easy to employ because it is noninvasive, non-painful and safe. It can probe the function of many different parts of the cerebral cortex, excite, inhibit and assess aspects of excitability. Its use will probably expand as it clearly supplements other tools that are used to examine human physiology, such as functional neuroimaging. PET or fMRI studies done during TMS might be able to identify the areas of brain activated by TMS and those regions functionally connected to the stimulated sites, allowing evaluation of *in vivo* anatomical connectivity³⁸. It will probably be used more particularly in the area of higher cortical function and in the assessment of drug effects. High frequency rTMS may have significant adverse effects, but when used safely, it will also find specific utility. As a therapeutic device, there are many possibilities, including some not explored yet. If it ultimately proves useful for treatment of depression, TMS may well become a standard medical tool. □

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The genome sequence of the plant pathogen *Xylella fastidiosa*

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Xylella fastidiosa is a fastidious, xylem-limited bacterium that causes a range of economically important plant diseases. Here we report the complete genome sequence of *X. fastidiosa* clone 9a5c, which causes citrus variegated chlorosis—a serious disease of orange trees. The genome comprises a 52.7% GC-rich 2,679,305-base-pair (bp) circular chromosome and two plasmids of 51,158 bp and 1,285 bp. We can assign putative functions to 47% of the 2,904 predicted coding regions. Efficient metabolic functions are predicted, with sugars as the principal energy and carbon source, supporting existence in the nutrient-poor xylem sap. The mechanisms associated with pathogenicity and virulence involve toxins, antibiotics and ion sequestration systems, as well as bacterium–bacterium and bacterium–host interactions mediated by a range of proteins. Orthologues of some of these proteins have only been identified in animal and human pathogens; their presence in *X. fastidiosa* indicates that the molecular basis for bacterial pathogenicity is both conserved and independent of host. At least 83 genes are bacteriophage-derived and include virulence-associated genes from other bacteria, providing direct evidence of phage-mediated horizontal gene transfer.

Citrus variegated chlorosis (CVC), which was first recorded in Brazil in 1987, affects all commercial sweet orange varieties¹. Symptoms include conspicuous variegations on older leaves, with chlorotic areas on the upper side and corresponding light brown lesions, with gum-like material on the lower side. Affected fruits are small, hardened and of no commercial value. A strain of *Xylella fastidiosa* was first identified as the causal bacterium in 1993 (ref. 2) and found to be transmitted by sharpshooter leafhoppers in 1996 (ref. 3). CVC control is at present limited to removing infected shoots by pruning, the application of insecticides and the use of healthy plants for new orchards. In addition to CVC, other strains of *X. fastidiosa* cause a range of economically important plant diseases including Pierce's disease of grapevine, alfalfa dwarf, phony peach disease, periwinkle wilt and leaf scorch of plum, and are also associated with diseases in mulberry, pear, almond, elm, sycamore, oak, maple, pecan and coffee⁴. The triply cloned *X. fastidiosa* 9a5c, sequenced here, was derived from the pathogenic culture 8.1b obtained in 1992 in Bordeaux (France) from CVC-affected Valencia sweet orange twigs collected in Macaubal (São Paulo, Brazil) on May 21, 1992 (ref. 2). Strain 9a5c produces typical CVC symptoms on inoculation into experimental citrus plants⁵, and into *Nicotiana tabacum* (S. A. Lopes, personal communication) and *Catharantus roseus* (P. Brant-Monteiro, personal communication)—two novel experimental hosts.

General features of the genome

The basic features of the genome are listed in Table 1 and a detailed map is shown in Fig. 1. The conserved origin of replication of the large chromosome has been identified in a region between the putative 50S ribosomal protein L34 and *gyrB* genes containing *dnaA*, *dnaN* and *recF*⁶. The *Escherichia coli* DnaA box consensus sequence TTATCCACA is found on both DNA strands close to *dnaA*. In addition, there are typical 13-nucleotide (ACCACCAC-CACCA) and 9-nucleotide (two TTTCATTGG and two TTTTAT-TATT) sequences in other intergenic sequences of this region. This region is coincident with the calculated GC-skew signal inversion⁷. We have designated base 1 of the *X. fastidiosa* genome as the first T of the only TTTTAT sequence found between the ribosomal protein L34 gene and *dnaA*.

The overall percentage of open reading frames (ORFs) for which a putative biological function could be assigned (47%) was slightly below that for other sequenced genomes such as *Thermotoga maritima*⁸ (54%), *Deinococcus radiodurans*⁹ (52.5%) and *Neisseria*

*meningitidis*¹⁰ (53.7%). This may reflect the lack of previous complete genome sequences from phytopathogenic bacteria. Plasmid pXF1.3 contains only two ORFs, one of which encodes a replication-associated protein. Plasmid pXF51 contains 64 ORFs, of which 5 encode proteins involved in replication or plasmid stability and 20 encode proteins potentially involved in conjugative transfer. One ORF encodes a protein similar to the virulence-associated protein D (VapD), found in many other bacterial pathogens¹¹. Four regions of pXF51 present significant DNA similarity to parts of transposons found in plasmids from other bacteria, suggesting interspecific horizontal exchange of genetic material.

The principal paralogous families are summarized in Table 2. The complete list of ORFs with assigned function is shown in Table 3. Seventy-five proteins present in the 21 completely sequenced genomes in the COG database¹² (as of 15th March 2000) were also found in *X. fastidiosa*. Each of these sequences was used to

Table 1 General features of the *Xylella fastidiosa* 9a5c genome

Main chromosome	
Length (bp)	2,679,305
G+C ratio	52.7%
Open reading frames (ORFs)	2,782
Coding region (% of chromosome size)	88.0%
Average ORF length (bp)	799
ORFs with functional assignment	1,283
ORFs with matches to conserved hypothetical proteins	310
ORFs without significant data base match	1,083
Ribosomal RNA operons	2
	(16SrRNA-Ala-TGC-tRNA-Ile-GAT-tRNA-23SrRNA-5SrRNA)
tRNAs	49 (46 different sequences corresponding to all 20 amino acids)
tmRNA	1
Plasmid pXF51	
Length (bp)	51,158
G+C ratio	49.6%
Open reading frames (ORFs)	64
Protein coding region (% of plasmid size)	86.9%
ORFs with functional assignment	30
ORFs with matches to conserved hypothetical proteins	8
ORFs without significant data base match	24
Plasmid pXF1.3	
Length (bp)	1,285
G+C ratio	55.6%
Open reading frames (ORFs)	2
ORFs with functional assignment	1

Table 2 Largest families of paralogous genes

Family (total number of families = 312)	Number of genes (total number of genes = 853)
ATP-binding subunits of ABC transporters	23
Reductases/dehydrogenases	12
Two-component system, regulatory proteins	12
Hypothetical proteins	10
Transcriptional regulators	9
Fimbrial proteins	9
Two-component system, sensor proteins	9

generate a phylogenetic tree of the 22 organisms. In 69% of such trees, *X. fastidiosa* was grouped with *Haemophilus influenzae* and *E. coli*, consistent with a phylogenetic analysis undertaken with the 16S rRNA gene¹³.

One ORF, a cytosine methyltransferase (XF1774), is interrupted by a Group II intron. The intron was identified on the basis of the presence of a reverse transcriptase-like gene (as in other Group II introns), conserved splice sites, conserved sequence in structure V and conserved elements of secondary structure¹⁴. Group II introns are rare in prokaryotes, but have been found in different evolutive lineages including *E. coli*, cyanobacteria and proteobacteria¹⁵.

Transcription, translation and repair

The basic transcriptional and translational machinery of *X. fastidiosa* is similar to that of *E. coli*¹⁶. Recombinational repair, nucleotide and base-excision repair, and transcription-coupled repair are present with some noteworthy features. For example, no photolyase was found, indicating exclusively dark repair. Although the main genes of the SOS pathway, *recA* and *lexA*, are present, ORFs corresponding to the three DNA polymerases induced by SOS in *E. coli* (DNA polymerases II, IV and V)¹⁷ are missing, indicating that the mutational pathway itself may be distinct.

Energy metabolism

Even though *X. fastidiosa* is, as its name suggests, a fastidious organism, energy production is apparently efficient. In addition to all the genes for the glycolytic pathway, all genes for the tricarboxylic acid cycle and oxidative and electron transport chains are present. ATP synthesis is driven by the resulting chemiosmotic proton gradient and occurs by an F-type ATP synthase. Fructose, mannose and glycerol can be utilized in addition to glucose in the glycolytic pathway. There is a complete pathway for hydrolysis of cellulose to glucose, consisting of 1,4- β -cellobiosidase, endo-1,4- β -glucanase and β -glucosidase, suggesting that cellulose breakdown may supplement the often low concentrations of monosaccharides in the xylem¹⁸. Two lipases are encoded in the genome, but there is no β -oxidation pathway for the hydrolysis of fatty acids, presumably precluding their utilization as an alternative carbon and energy source. Likewise, although enzymes required for the breakdown of threonine, serine, glycine, alanine, aspartate and glutamate are present, pathways for the catabolism of the other naturally occurring amino acids are incomplete or absent.

The gluconeogenesis pathway appears to be incomplete. Phosphoenolpyruvate carboxykinase and the gluconeogenic enzyme fructose-1,6-bisphosphatase, which are required to bypass the irreversible step in glycolysis, are not present. The absence of the first is compensated by the presence of phosphoenolpyruvate synthase and malate oxidoreductase, which together can generate phosphoenolpyruvate from malate. There appears, however, to be no known compensating pathway for the absence of fructose-1,6-bisphosphatase. It is possible that among the large number of unidentified *X. fastidiosa* genes there are non-homologous genes that compensate for steps in such critical pathways. Barring this possibility, however, the absence of a functional gluconeogenesis pathway implies a strict dependence on carbohydrates both as a

source of energy and anabolic precursors. The glyoxylate cycle is absent and the pentose phosphate pathway is incomplete. In the latter pathway, genes for neither 6-phosphogluconic dehydrogenase nor transaldolase were identified.

Small molecule metabolism

X. fastidiosa exhibits extensive biosynthetic capabilities, presumably an absolute requirement for a xylem-dwelling bacterium. Most of the genes found in *E. coli* necessary for the synthesis of all amino acids from chorismate, pyruvate, 3-phosphoglycerate, glutamate and oxaloacetic acid¹⁶ were identified. However, some genes in *X. fastidiosa* are bi-functional, such as phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphatase (XF2213), aspartokinase/homoserine dehydrogenase I (XF2225), imidazole-glycerolphosphate dehydratase/histidinol-phosphate phosphatase (XF2217) and a new diaminopimelate decarboxylase/aspartate kinase (XF1116) that would catalyse the first and the last steps of lysine biosynthesis. In addition, the gene for acetylglutamate kinase (XF1001) has an acetyltransferase domain at its carboxy-terminal end that would compensate for the missing acetyltransferase in the arginine biosynthesis pathway. Other missing genes include phosphoserine phosphatase, cystathionine β -lyase, homoserine *O*-succinyltransferase and 2,4,5-methyltetrahydrofolate-homocysteine methyltransferase. The first two enzymes are also absent in the *Bacillus subtilis* genome, the third is absent in *Haemophilus influenzae* and the fourth is missing in both genomes¹². We thus presume that alternative, unidentified enzymes complete the biosynthetic pathways in these organisms and in *X. fastidiosa*.

The pathways for the synthesis of purines, pyrimidines and nucleotides are all complete. *X. fastidiosa* is also apparently capable of both synthesizing and elongating fatty acids from acetate. Again, however, some *E. coli* enzymes were not found, such as holo acyl-carrier-protein synthase (also absent in *Synechocystis* sp., *H. influenzae* and *Mycoplasma genitalium*) and enoyl-ACP reductase (NADPH) (*FabI*) (also absent from *M. genitalium*, *Borrelia burgdorferi* and *Treponema pallidum*)¹².

X. fastidiosa appears to be capable of synthesizing an extensive variety of enzyme cofactors and prosthetic groups, including biotin, folic acid, pantothenate and coenzyme A, ubiquinone, glutathione, thioredoxin, glutaredoxin, riboflavin, FMN, FAD, pyrimidine nucleotides, porphyrin, thiamin, pyridoxal 5'-phosphate and lipolate. In a number of the synthetic pathways, one or more of the enzymes present in *E. coli* are absent, but this is also true for at least one other sequenced Gram-negative bacterial genome in each case¹². We therefore again infer that the missing enzymes are either not essential or replaced by unknown proteins with novel structures.

Transport-related proteins

A total of 140 genes encoding transport-related proteins were identified, representing 4.8% of all ORFs. For comparison, *E. coli*, *B. subtilis* and *M. genitalium* have around 10% of genes encoding transport proteins, whereas *Helicobacter pylori*, *Synechocystis* sp. and *Methanococcus jannaschii* have 3.5–5.4% (ref. 19). Transport systems are central components of the host–pathogen relationship (Fig. 2). There are a number of ion transporters and transporters for the uptake of carbohydrates, amino acids, peptides, nitrate/nitrite, sulphate, phosphate and vitamin B12. Many different transport

Figure 1 Linear representation of the main chromosome and plasmids pXF51 and pXF1.3 of the *Xylella fastidiosa* genome. Genes are coloured according to their biological role. Arrows indicate the direction of transcription. Genes with frameshift and point mutations are indicated with an X. Ribosomal RNA genes, the tmRNA, the principal repeats, prophages and the group II intron are indicated by coloured lines. Transfer RNAs are identified by a single letter identifying the amino acid. Pie chart represents the distribution of the number of genes according to biological role. The numbers below protein-producing genes correspond to gene IDs.

families are represented and include both small and large mechanosensitive conductance ion channels, a monovalent cation:proton antiporter (CAP-2) and a glycerol facilitator belonging to the major intrinsic protein (MIP) family. In addition, 23 ABC transport systems comprising 41 genes can be identified. *X. fastidiosa* appears to possess a phosphotransferase system (PTS) that typically mediates small carbohydrate uptake. There are both the enzyme I and HPr components of this system, as well as a gene supposedly involved in its regulation (*pstK* or *hprK*); however, there is no PTS permease—an essential component of the phosphotransferase complex. The functionality of the system therefore remains in question.

There are five outer membrane receptors, including siderophores, ferrichrome-iron and haemin receptors, which are all associated with iron transport. The energizing complexes, TonB–ExbB–ExbD and the paralogous TolA–TolR–TolQ, essential for the functioning of the outer membrane receptors, are also present. In all, 67 genes encode proteins involved in iron metabolism. We propose that in *X. fastidiosa* the uptake of iron and possibly of other transition metal ions such as manganese causes a reduction in essential micronutrients in the plant xylem, contributing to the typical symptoms of leaf variegation.

The *X. fastidiosa* genome encodes a battery of proteins that mediate drug inactivation and detoxification, alteration of potential drug targets, prevention of drug entry and active extrusion of drugs and toxins. These include ABC transporters and transport processes driven by a proton gradient. Of the latter, eight belong to the hydrophobe/amphiphile efflux-1 (HAE1) family, which act as multidrug resistance factors.

Adhesion

X. fastidiosa is characteristically observed embedded in an extracellular translucent matrix *in planta*²⁰. Clumps of bacteria form within the xylem vessels leading to their blockage and symptoms of the disease such as water-stress leaf curling. We deduce, from our analysis of the complete genome sequence, that the matrix is composed of extracellular polysaccharides (EPSs) synthesized by enzymes closely related to those of *Xanthomonas campestris* pv *campestris* (Xcc) that produce what is commercially known as xanthan gum. In comparison with Xcc, however, we did not find *gumI* (encoding glycosyltransferase V, which incorporates the terminal mannose), *gumL* (encoding ketolase which adds pyruvate to the polymer) or *gumG* (encoding acetyltransferase which adds acetate), suggesting that *Xylella* gum may be less viscous than its *Xanthomonas* counterpart.

Positive regulation of the synthesis of extracellular enzymes and EPS in *Xanthomonas* is effected by proteins coded by the *rfp* (regulation of pathogenicity factors) gene cluster²¹. Mutations in any of these genes in *Xanthomonas* results in failure to synthesize the EPS. In consequence, the strain becomes non-pathogenic²¹. *X. fastidiosa* contains genes that encode RpfA, RpfB, RpfC and RpfF, suggesting that both bacteria may regulate the synthesis of pathogenic EPS factors through similar mechanisms.

Fimbria-like structures are readily apparent upon electron microscopical observation of *X. fastidiosa* within both its plant and insect hosts²². Because of the high velocity of xylem sap passing through narrow portions of the insect foregut, fimbria-mediated attachment may be essential for insect colonization. Indeed, in the insect mouthparts the bacteria are attached in ordered arrays, indicating specific and polarized adhesion²³. In addition, fimbriae are thought to be involved in both plant–bacterium and bacterium–bacterium interactions during colonization of the xylem itself. We identified 26 genes encoding proteins responsible for the biogenesis and function of Type 4 fimbria filaments. This type of fimbria is found at the poles of a wide range of bacterial pathogens where they act to mediate adhesion and translocation along epithelial surfaces²⁴. The genes include *pilS* and *pilR* homologues, which encode a two-component

system controlling transcription of fimbrial subunits, presumably in response to host cues, and *pilG*, *H*, *I*, *J* and *chpA*, which encode a chemotactic system transducing environmental signals to the pilus machinery.

In addition to the EPS and fimbriae, which are likely to have central roles in the clumping of bacteria and in adhesion to the xylem walls, we also identified outer membrane protein homologues for afimbrial adhesins. Although fimbrial adhesins are well characterized as crucial virulence factors in both plant and human pathogens²⁵, afimbrial adhesins, which are directly associated with the bacterial cell surface, have been hitherto associated only with human and animal pathogens, where they promote adherence to epithelial tissue. Of the three putative adhesins of this kind identified in *X. fastidiosa*, two exhibit significant similarity to each other (XF1981, XF1529) and to the *hsf* and *hia* gene products of *H. influenzae*²⁶. The third (XF1516) is similar to the *uspA1* gene product of *Moraxella catarrhalis*²⁷. All these proteins share the common C-terminal domain of the autotransporter family²⁸. Direct experimentation will be required to establish whether these adhesins promote binding to plant cell structures or components of the insect vector foregut, or both. Nevertheless, their presence in the *X. fastidiosa* genome adds to the increasing evidence for the generality of mechanisms of bacterial pathogenicity, irrespective of the host organism²⁹.

We also identified three different haemagglutinin-like genes. Again, similar genes have not previously been identified in plant pathogens. These genes (XF2775, XF2196, XF0889) are the largest in the genome and exhibit highest similarity to a *Neisseria meningitidis* putative secreted protein¹⁰.

Intervessel migration

Movement between individual xylem vessels is crucial for effective colonization by *X. fastidiosa*. For this to occur, degradation of the pit membrane of the xylem vessel is required. Of the known pectolytic enzymes capable of this function, a polygalacturonase precursor and a cellulase were identified, although the former contains an authentic frameshift. These genes exhibited highest similarity to orthologues in *Ralstonia solanacearum*—which causes wilt disease in tomatoes—where the polygalacturonase genes are required for wild-type virulence.

Toxicity

We identified five haemolysin-like genes: haemolysin III (XF0175), which belongs to an uncharacterized protein family, and four others (XF0668, XF1011, XF2407, XF2759) which belong to the RTX toxin family that contains tandemly repeated glycine-rich nonapeptide motifs at the C-terminal domain. One of these ORFs is closely related to bacteriocin, an RTX toxin also found in the plant bacterium *Rhizobium leguminosarum*³⁰. RTX or RTX-like proteins are important virulence factors widely distributed among Gram-negative pathogenic bacteria³¹.

There are two Colicin-V-like precursor proteins. Colicin V is an antibacterial polypeptide toxin produced by *E. coli*, which acts against closely related sensitive bacteria³². The precursors consist of 102-amino-acid peptides (XF0262, XF0263) that have the typical conserved leader 15-amino-acid motif, and have some similarity with Colicin V from *E. coli* at the remaining C-terminal portion. The necessary apparatus for Colicin biosynthesis and secretion is also present. Interestingly, in *E. coli* most of the genes necessary for biogenesis and export of Colicin V are in a gene cluster present in a plasmid, whereas in *X. fastidiosa* these genes are dispersed in the chromosome.

We found four genes that may function in polyketide biogenesis: polyketide synthase (PKS), pteridine-dependent deoxygenase, daunorubicin C-13 ketoreductase and a NonF-related protein. These genes belong to the synthesis pathways of frenolicin, rapamycin, daunorubicin and nonactin, respectively. These pathways

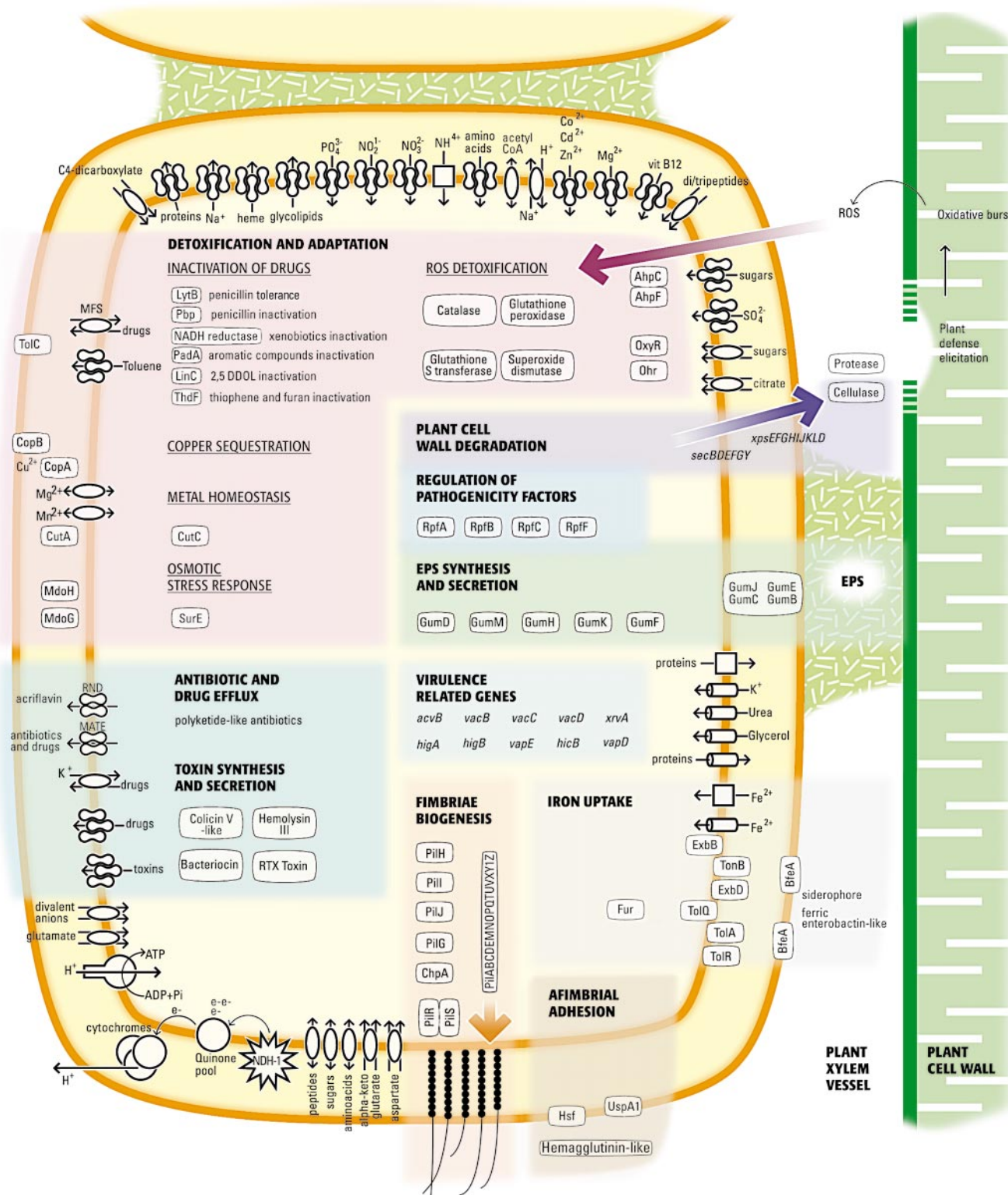


Figure 2 A comprehensive view of the biochemical processes involved in *Xylella fastidiosa* pathogenicity and survival in the host xylem. The principal functional categories are shown in bold, and the bacterial genes and gene products related to that function are arranged within the coloured section containing the bold heading. Transporters are indicated as follows: cylinders, channels; ovals, secondary carriers, including the MFS family; paired dumbbells, secondary carriers for drug extrusion; triple dumbbells, ABC transporters; bulb-like icon, F-type ATP synthase; squares, other transporters. Icons with two arrows

represent symporters and antiporters (H^+ or Na^+ porters, unless noted otherwise). 2,5DDOL, 2,5-dichloro-2,5-cyclohexadiene-1,4-diol; EPS, exopolysaccharides; MATE, multi-antimicrobial extrusion family of transporters multidrug efflux gene (XF2686); MFS, major facilitator superfamily of transporters; Pbp, β -lactamase-like penicillin-binding protein (XF1621); RND, resistance-nodulation-cell division superfamily of transporters; ROS, reactive oxygen species.

include many more enzymes, which we did not find; however, some of the genes listed lie close to ORFs without significant database matches, suggesting that at least one (as yet undiscovered) polyketide pathway may be functional.

Prophages

Bacteriophages can mediate the evolution and transfer of virulence factors and occasional acquisition of new traits by the bacterial host. Because as much as 7% of the *X. fastidiosa* genome sequenced corresponds to double-stranded (ds) DNA phage sequences, mostly from the Lambda group, we suspect that this route may have been of particular importance for this bacterium. It is noteworthy that a very high percentage of phage-related sequences has also been detected in a second vascular-restricted plant pathogen, *Spiroplasma citri*³³. We identified four regions, with a high density of ORFs homologous to phage sequences, that we considered to be prophages, in addition to isolated phage sequences dispersed throughout the genome. Two of these prophages (each ~42 kbp, designated Xfp1 and Xfp2) are similar to each other, lie in opposite orientations in distinct regions and appear to belong to the dsDNA, tailed-phage group. Both appear to contain most of the genes responsible for particle assembly, although we know of no reports of phage particle release from *X. fastidiosa* cultures. In prophage Xfp1, we found two ORFs between tail genes V and W that are similar to ORF118 and *vapA* from the virulence-associated region of the animal pathogen *Dichelobacter nodosus*, which by homology encode a killer and a suppressor protein³⁴. Interestingly, in prophage Xfp2, we found two other ORFs also between tail genes V and W that are similar to hypothetical ORFs of *Ralstonia eutropha* transposon Tn4371 (ref. 35). The other two identified prophages, Xfp3 and Xfp4, are also similar in sequence to each other and to the *H. influenzae* cryptic prophage ϕ flu (ref. 36). They both contain a 14,317-bp exact repeat. Few particle-assembly genes were found in these regions, suggesting that these prophages are defective. An ORF similar to *hicB* from *H. influenzae*, a component of the major pilus gene cluster in some isolates, was found in Xfp4 (ref. 37).

The presence of virulence-associated genes from other organisms within the prophage sequences is strong evidence for a direct role for bacteriophage-mediated horizontal gene transfer in the definition of the bacterial phenotype.

Absence of avirulence genes

Phytopathogenic bacteria generally have a limited host range, often confined to members of a single species or genus. This specificity is defined by the products of the so-called avirulence (*avr*) genes present in the pathogen, which are injected directly into host cells, on infection, through a type III secretory system^{38–40}. BLAST⁴¹ searches with all known *avr* and type III secretory system sequences failed to identify genes encoding proteins with significant similarities in the genome of *X. fastidiosa*. Although the variability of *avr* genes amongst bacteria might account for this apparent lack, the high level of similarity of some components of the type III secretory system argues against this. We suspect that these genes are, in fact, not required because of the insect-mediated transmission and vascular restriction of the bacterium that obviates the necessity of host cell infection. Furthermore, if the differing host ranges of *X. fastidiosa* are molecularly defined, this may be by a quite different mechanism not involving *avr* proteins.

Conclusions

Before the elucidation of its complete genome sequence, very little was known of the molecular mechanisms of *X. fastidiosa* pathogenicity. Indeed, this bacterium was probably the least characterized of all organisms that have been fully sequenced. Our complete genetic analysis has determined not only the basic metabolic and replicative characteristics of the bacterium, but also a number of potential

pathogenicity mechanisms. Some of these have not previously been postulated to occur in phytopathogens, providing new insights into the generality of these processes. Indeed, the availability of this first complete plant pathogen genome sequence will now allow the initiation of the detailed comparison of animal and plant pathogens at the whole-genome level. In addition, the information contained in the sequence should provide the basis for an accelerated and rational experimental dissection of the interactions between *X. fastidiosa* and its hosts that might lead to fresh insights into potential approaches to the control of CVC. □

Methods

The sequencing and analysis in this project were carried out by a network of 34 biology laboratories and one bioinformatics centre. The network is called the Organization for Nucleotide Sequencing and Analysis (ONSA)⁴², and is entirely located in the state of São Paulo, Brazil.

Sequencing and assembly

The sequence was generated using a combination of ordered cosmid and shotgun strategies⁴³. A cosmid library was constructed, providing roughly 15-fold genome coverage, containing 1,056 clones with average insert size of 40 kilobases (kb). High-density colony filters of the library were made, and a physical map of the genome was constructed using a strategy of hybridization without replacement⁴⁴. A total of 113 cosmid clones was selected for sequencing on the basis of the hybridization map and end-sequence analysis. The cosmid sequences were assembled into 15 contigs covering 90% of the genome. Additionally, shotgun libraries with different insert sizes (0.8–2.0 kb and 2.0–4.5 kb) were constructed from nebulized or restricted genomic DNA cloned into plasmids, and sequenced to achieve a 3.74-fold coverage of high-quality sequence (29,140 reads). Most of the sequencing was performed with BigDye terminators on ABI Prism 377 DNA sequencers.

Cosmid and shotgun sequences were assembled into six contigs. We identified sequence gaps by linking information from forward and reverse reads, and closed either by primer walking or insert subcloning. The remaining physical gaps were closed by combinatorial PCR and by lambda clones selected from a λ Dash library by end-sequencing. The collinearity between the genome and the obtained sequence was confirmed by digestion of genomic DNA with *AscI*, *NotI*, *SfiI*, *SmaI* and *SrfI*, followed by comparison of the digestion pattern with the electronic digestion of the generated sequence. In addition, sequences from both ends of most cosmid clones and 236 λ clones were used to confirm the orientation and integrity of the contigs. The sequence was assembled using phred+phrap+consed⁴⁵. All consensus bases have quality with Phred value of at least 20. There are no unexplained high quality discrepancies, each consensus base is confirmed by at least one read from each strand, and the overall error estimate is less than 1 in every 10,000 bases.

ORF prediction and annotation

ORFs were determined using glimmer 2.0 (ref. 46) and the glimmer post-processor RBSfinder (S. L. Salzberg, personal communication). A few ORFs were found by hand guided by BLAST⁴¹ results. Annotation was carried out in a cooperative way, mostly by comparison with sequences in public databases, using BLAST⁴¹ and tRNAscan-SE (ref. 47) and was based on the functional categories for *E. coli*⁴⁸. Only one tmRNA was located (K. Williams, personal communication). To help annotate transport proteins, we built a custom BLAST⁴¹ database using sequences from <http://www-biology.ucsd.edu/~msaier/transport/toc.html> and compared our ORFs with these sequences. Phylogenetic trees for conserved COG⁴² were built using ClustalX⁴⁹ for multiple alignment and Phylip⁵⁰. Paralogous gene families (Table 2) were determined using BLASTX with the E-value cut-off equal to e^{-5} and such that at least 60% of the query sequence and at least 30% of the subject sequence were aligned.

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A large age for the pulsar B1757–24 from an upper limit on its proper motion

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The ‘characteristic age’¹ of a pulsar is usually considered to approximate its true age, but this assumption has led to some puzzling results, including the fact that many pulsars with small characteristic ages have no associated supernova remnants^{2,3}. The pulsar B1757–24 is located just outside the edge of a supernova remnant^{4–6}; the properties of the system indicate that the pulsar was born at the centre of the remnant with a substantial velocity, and that it has subsequently overtaken the expanding blast wave^{5–8}. With a characteristic age of 16,000 yr, the pulsar is expected⁸ to have a proper motion of 63–80 milliarcseconds (mas) per year. Here we report observations of the nebula surrounding the pulsar, which limit its proper motion to less than 25 mas yr^{–1}, implying a minimum age of 39,000 yr. A more detailed analysis argues that the true age may be as great as 170,000 yr, which is significantly larger than the characteristic age. We conclude from this result and other discrepancies associated with pulsars that characteristic ages greatly underestimate the true ages of pulsars.

The radio supernova remnant G5.4–1.2 is shown in Fig. 1: it has an approximately circular radio morphology of diameter about 35', but is brightest and has the flattest spectrum along its western edge^{6,8–10}. Approximately 5' beyond this bright western rim is the 125-ms pulsar B1757–24^{4–6}, whose rotational frequency, ν , and frequency derivative, $\dot{\nu}$, can be used to derive a characteristic age, $\tau = \nu/2\dot{\nu} \approx 16$ kyr. Provided that a pulsar's initial frequency is much larger than ν , that the pulsar slows down by magnetic dipole braking, and that its magnetic dipole moment and moment of inertia do not evolve with time, then $\tau \approx t_p$, where t_p is the pulsar's true age¹.

Surrounding the pulsar is the radio nebula G5.27–0.90, whose properties are consistent with it being powered by the pulsar's relativistic wind^{6,8}. G5.27–0.90 has a cometary morphology with a tail pointing back towards the centre of G5.4–1.2, an appearance which strongly suggests that the pulsar is travelling away from the centre of G5.4–1.2 at high velocity. The characteristic age of B1757–24 and its positional offset from the centre of G5.4–1.2 together imply a proper motion of 63–80 mas yr^{–1} (ref. 8). For a distance to the system of 5 kpc (ref. 8), this corresponds to a transverse velocity for the pulsar in the range 1,500–1,900 km s^{–1}, significantly higher than typical pulsar velocities of about 500 km s^{–1} (refs 11 and 12).

To look for this expected proper motion, we have undertaken multi-epoch observations of G5.27–0.90 with the Very Large Array (VLA). Our observations are summarized in Table 1; the two epochs are separated by 6.7 yr. The two data sets were processed in identical fashion, with results shown in Fig. 2. If G5.27–0.90 is associated with the pulsar, then the expected shift of the nebula between these two epochs is 422–526 mas in a westward direction; it is immediately apparent, even by eye, that motion of this magnitude is not observed. The two epochs can be compared in a more quantitative fashion using the approach described in the legend to Fig. 2; we find an offset between the two epochs (in the sense of [epoch 2]–[epoch 1]) of $\Delta(\text{right ascension, RA}) = -39 \pm 41$ mas and

$\Delta(\text{declination, Dec}) = -9 \pm 34$ mas, where contributions from both the noise in the images and estimated errors in the phase calibration of the data have been combined in quadrature. We can thus rule out the proper motion which would be expected if $t_p = \tau$ at the 11–14 σ level, independent of the distance adopted to either the pulsar or the supernova remnant (SNR).

We now consider various explanations for this surprising discrepancy, and show that the most reasonable explanation is that $t_p \gg \tau$. In the following discussion, we only consider proper motion in a westward direction (as implied by the morphology of G5.27–0.90), and adopt a 5- σ upper limit on any shift between epochs of 166 mas corresponding to a maximum proper motion of 24.8 mas yr^{–1}. For a distance $d = 5d_5$ kpc and a projected velocity of the nebula $V_n = 1,000v_n$ km s^{–1}, this allows us to derive an upper limit $v_n < 0.59d_5$.

One possible explanation for this unexpectedly low velocity is that the pulsar is moving at $V_p \approx 1,500$ km s^{–1} $\approx 2.5v_n$, but its associated nebula, sensitive to its surroundings, does not track the ballistic motion of the pulsar on a one-to-one basis. The distance, r_w , between the pulsar and the head of the bow-shock is determined by pressure balance between the pulsar wind and the ram pressure produced by the pulsar's motion, such that^{6,13}:

$$\eta\dot{E}/4\pi r_w^2 c = \frac{4}{9}\rho V_p^2 \quad (1)$$

where ρ is the mass density of the ambient medium and η is the fraction of the pulsar's spin-down luminosity, $\dot{E} \equiv -4\pi^2 I \nu \dot{\nu} = 2.6 \times 10^{36}$ erg s^{–1}, which goes into powering its relativistic wind ($\eta < 1$). Using the pulsar's position as measured in late 1999 (see legend to Fig. 2), we find that $r_w = 0.036d_5$ pc for the second epoch. Assuming that $v_p = 2.5v_n$ (where $V_p = 1,000v_p$ km s^{–1}), we can then infer that r_w must have decreased by at least 20% between epochs 1 and 2. A decrease in $\eta\dot{E}$ by a similar factor can produce this effect. However, comparison of pulsar timing parameters for the time of epoch 2 (A. G. Lyne, personal communication) with those published before epoch 1 (ref. 5) shows no significant change in $\dot{E}$, and a change in η seems unlikely given that changes in the efficiency of pulsar winds, if they occur at all, most probably occur on timescales of about 10⁴–10⁶ yr (refs 14 and 15).

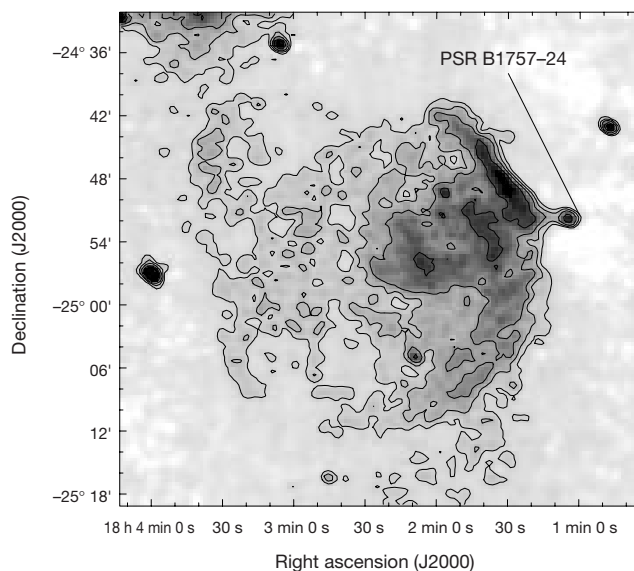


Figure 1 Radio emission from the supernova remnant G5.4–1.2. The image corresponds to archival VLA data at an observing wavelength of 90 cm; contours are at levels of 10, 25, 50, 100, 150 and 200 mJy beam^{–1}, and the peak intensity is 150 mJy beam^{–1}. The resolution of the image is 60'' $\times$ 45''. The pulsar (whose position is marked) is at the head of the compact nebula G5.27–0.90 to the west of the supernova remnant.

A reasonable alternative is that the pulsar is propagating into a density gradient so that ρ is increasing as a function of time, causing a decrease in r_w . However, an increasing density should result in a change in the morphology or surface brightness of the leading edge of G5.27–0.90. In fact, the structure and brightness of the head of G5.27–0.90 is remarkably similar between the two epochs, and also in archival 6-cm VLA data taken at various epochs between 1985 and 1999. There is thus no evidence from the morphology of G5.27–0.90 for a change in ambient conditions between the two epochs.

It thus seems most likely that r_w has not changed between the two epochs, implying that $V_p = V_n \ll 1,500 \text{ km s}^{-1}$. In this case, demanding that $t_p \approx \tau$ would mean that the pulsar had only travelled $7'$ over its lifetime, inconsistent with its separation of $16.1'–20.6'$ from the apparent centre of the SNR (ref. 8) and thus arguing against a physical association between the two objects. Although such chance coincidence along the line-of-sight is not impossible¹⁶, the strong morphological evidence for an association^{5–8,10} leads us to conclude that $t_p > 39–50 \text{ kyr} \gg \tau$. This result depends only on assuming that $V_p = V_n$, and that the pulsar was born near the SNR's apparent centre.

Another approach is to let the age of the system, the ambient

density and the transverse velocity of the pulsar all be free parameters. We can then solve for these unknowns using the three characteristic length scales of the system, namely the radius of the SNR ($R_s = 25d_5 \text{ pc}$), the stand-off distance of the bow-shock ($r_w = 0.036d_5 \text{ pc}$) and the offset of the pulsar from the SNR's centre ($R_p = (23.4 - 30.0)d_5 \text{ pc}$). To determine how the unknown quantities relate to R_s , we first need to determine the SNR's evolutionary state. Using our upper limit $v_p < 0.59d_5$ and the value measured for r_w , equation (1) implies a particle density $n_1 > n_0 = 0.23\eta d_5^4 \text{ cm}^{-3}$. Assuming that the SNR is expanding into a similar environment, it will be in the radiative (or 'snow-plough') phase of its evolution^{7,17} if $t_p > t_R = 13E_{51}^{3/14} n_1^{-4/7} \text{ kyr}$, where $E_{51} \times 10^{51} \text{ erg}$ is the energy in the supernova explosion and assuming solar abundances in the interstellar medium. The lower limits we have already computed on n_1 and t_p ensure that the SNR is indeed in the radiative phase; the radius (in pc) of the SNR is then given by^{7,17}:

$$R_s = 7.0E_{51}^{0.221} n_1^{-0.257} t_3^{0.3} \quad (2)$$

where $t_p = t_3 \text{ kyr}$. Meanwhile, ballistic motion of the pulsar away from the centre of the SNR gives a distance travelled (in pc) by the pulsar:

$$R_p = 1.02v_p t_3 \quad (3)$$

and we can rewrite equation (1) to show that:

$$r_w = 0.010\eta^{1/2} n_1^{-1/2} v_p^{-1} \quad (4)$$

Equations (2), (3) and (4) can be solved simultaneously to give $v_p = (0.17 - 0.25)E_{51}^{-1.04} \eta^{1.21} d_5^{2.3}$, $n_1 = (1.3 - 2.6)E_{51}^{2.08} \eta^{-1.42} d_5^{-6.6}$ and $t_3 = (93 - 170)E_{51}^{1.04} \eta^{-1.21} d_5^{-2.3}$. Self-consistency with the requirement that $n_1 > n_0$ then implies $d_5 < 1.3E_{51}^{2.08} \eta^{-2.42}$, which, combined with⁸ $d_5 > 0.9$ (from H I absorption) and $d_5 \approx 0.9$ (from the pulsar's dispersion measure) justifies the choices $d_5 = 1$ and $E_{51} = 1$.

Thus two different approaches—using either the limits on proper motion, or the morphology of the system—both imply a much older and more slowly moving pulsar than results⁶ when one assumes $t_p = \tau$. We note that a similar age and velocity to those we have just estimated were derived before the characteristic age of the pulsar was known, by assuming $n_1 = 1$ and then solving together the equivalents of equations (2) and (3) (ref. 7).

For a pulsar of constant magnetic dipole moment and moment of inertia, it can be shown that¹:

$$\frac{t_p}{\tau} = \frac{2}{n-1} \left[1 - \left(\frac{v}{v_0} \right)^{n-1} \right] \quad (5)$$

where v_0 is the pulsar's initial rotational frequency and n is the 'braking index', defined by $\dot{v} = -Kv^n$ (where K is a constant), and expected to be $n = 3$ for a dipole rotating *in vacuo*. Our lower limit on t_p/τ from proper motion alone implies that $n < 1.83$, while the value derived from the morphology of the system argues that $n < 1.33$. These values are significantly less than those derived for young pulsars^{18,19}, but are similar to $n = 1.4 \pm 0.2$ observed for the Vela pulsar²⁰, a pulsar which also resembles the pulsar PSR B1757–24 in its value of τ and its tendency to 'glitch'²¹. One alternative possibility is that the magnetic field of PSR B1757–24 is growing as a function of time^{22,23}. In this case equation (5) does not hold and K is no longer a constant; τ is then an approximate timescale for field growth.

Further data can support the conclusion that PSR B1757–24 is much older than $\tau = v/2\dot{v}$: multi-epoch very long baseline interferometry (VLBI) observations are expected to show westward motion of the pulsar at the level of about 10 mas yr^{-1} . X-ray observations of SNR G5.4–1.2 should show emission characteristic of an older rather than young SNR, and a braking index for PSR B1757–24 measured from timing data would be expected to lie in

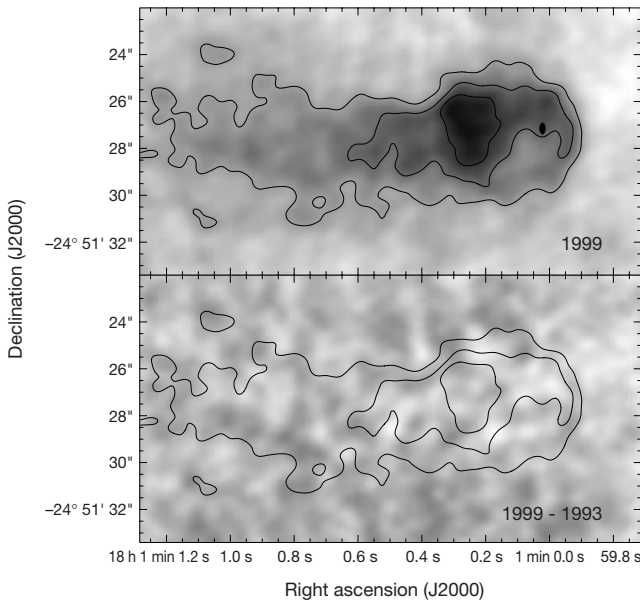


Figure 2 Radio emission from the western tip of the pulsar-powered nebula G5.27–0.90. The upper panel represents the 1999 image of G5.27–0.90, with corresponding contour levels at 0.75, 1.5 and $2.25 \text{ mJy beam}^{-1}$. The ellipse near the head of the nebula marks the position of PSR B1757–24, (J2000) RA 18 h 01 min 00.023(2) s, Dec $-24^\circ 51' 27.15(5)''$, (where parenthesized value indicates uncertainty in last digit), which was determined by 20-cm A-array observations in August 1999 (uncertainties in the position of the pulsar are a factor of five smaller than the extent of the ellipse). The lower panel shows the difference between the 1999 and 1993 images, on which are superimposed the same contour levels as in the upper panel. To compare the two epochs in a quantitative fashion, we took the deconvolved model from each epoch, computed its two-dimensional Fourier transform, and then samples this transform with the transfer function corresponding to the $u-v$ coverage of, that is, spatial frequencies measured by, the 1993 data. Assuming fidelity in the original deconvolution, this results in two data sets which have identical $u-v$ coverage and sensitivity, and differ only in the actual brightness distribution between the two epochs. We searched for proper motion between these two images using the MIRIAD task IMDIFF²⁸, which finds the shift in x and y that minimizes²⁹ the r.m.s. noise of the difference map between the two data sets. Shifts corresponding to a non-integral number of pixels were computed using cubic convolution interpolation³⁰. To estimate the uncertainties associated with this approach, we repeated this comparison 40 times, each time adding gaussian noise of r.m.s. $30 \mu\text{Jy beam}^{-1}$ (corresponding to the sensitivity of the epoch 1 data) to one of the images.

Table 1 Very Large Array observations of the pulsar-powered nebula G5.27–0.90

	Epoch 1	Epoch 2
Date observed	1993 Feb. 02	1999 Oct. 23
Array configuration	BnA	BnA
On-source integration time (h)	3.3	2.8
Centre frequency (GHz)	8.44	8.46
Bandwidth (MHz)	75	100

These observations were used for proper motion measurements. Both observations used the same pointing centre, namely (J2000) right ascension (RA) 18 h 01 min 00.035 s, declination (Dec) $-24^{\circ} 51' 27.260''$. Absolute flux densities were determined using observations of the source 3C286, while antenna gains were calibrated using observations every 15–20 min of the extragalactic source PMN J1751–2524, displaced $2.1''$ from G5.27–0.90. The resulting images were formed using natural weighting and multi-frequency synthesis with 100-mas pixels, deconvolved using a maximum entropy algorithm, and then smoothed with a circular gaussian of FWHM $0.90''$.

the range predicted above. The low braking index of the Vela pulsar²⁰, discrepant pulsar–SNR ages in other pulsar–SNR associations^{23,24}, and the fact that many pulsars with small characteristic ages have no associated SNR (refs 2 and 3), all suggest that PSR B1757–24 is not a statistical anomaly. If other pulsars are indeed older than they seem, our understanding of pulsar velocities, asymmetries in supernova explosions, the fraction of supernovae that produce pulsars, and the physics of neutron star structure and cooling must all be reconsidered^{12,25–27}. □

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Realignment of the flux-line lattice by a change in the symmetry of superconductivity in UPT₃

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In 1957, Abrikosov¹ described how quanta of magnetic flux enter the interior of a bulk type II superconductor. It was subsequently predicted that, in an isotropic superconductor, the repulsive forces between the flux lines would cause them to order in two dimensions, forming a hexagonal lattice². Flux-line lattices with different geometry can also be found in conventional (type II) superconductors³; however, the ideal hexagonal lattice structure should always occur when the magnetic field is applied along a hexagonal crystal direction⁴. Here we report measurements of the orientation of the flux-line lattice in the heavy-fermion superconductor⁵ UPT₃, for this special case. As the temperature is increased, the hexagonal lattice, which is initially aligned along the crystal symmetry directions, realigns itself with the anisotropic superconducting gap. The superconductivity in UPT₃ is unusual (even compared to unconventional oxide superconductors⁶) because the superconducting gap has a lower rotational symmetry than the crystal structure. This special feature enables our data to demonstrate clearly the link between the microscopic symmetry of the superconductivity and the mesoscopic physics of the flux-line lattice. Moreover, our observations provide a stringent test of the theoretical description of the unconventional superconductivity in UPT₃.

In a recent study⁷ of the magnetic borocarbide TmNi₂B₂C it was shown that a flux-line lattice (FLL) can align itself with a coexisting modulated magnetic structure. Here we present evidence that a FLL can also be aligned to the intrinsic symmetry of the superconducting order. In previous work on UPT₃, the FLL geometry was measured at low temperature as a function of field for magnetic fields directed parallel to a crystallographic *a* direction^{8,9}. The symmetry and orientation of the FLL for this field direction was found to be independent of the field strength, but a strong evolution of the distortion of the FLL geometry with field was observed. This has been interpreted to support a description of the superconductivity in terms of a two-component order parameter¹⁰. In the present

study we report a fundamental change in the symmetry of the FLL with temperature, when the field is applied parallel to the hexagonal *c* direction. Owing to the special choice of the hexagonal direction, the observed symmetry change cannot be explained by Fermi-surface anisotropy^{11,12}, which can otherwise provide a conventional explanation for the occurrence of different symmetry FLLs. The presence of this competing mechanism has complicated the interpretation of a previous study of the unconventional superconductor Sr_2RuO_4 (ref. 13).

Our measurements were performed on an annealed cylindrical single crystal, grown parallel to a crystallographic *a* direction by the Czochralski technique under ultrahigh vacuum. Conventional neutron diffraction detected no crystal mosaicity within an instrumental resolution of 0.35° (full-width at half-maximum), and the crystal's superconducting properties were measured to be similar to other high-quality samples^{14,15}.

The FLL was studied by small-angle neutron scattering with the D22 instrument at the Institut Laue Langevin, France. The neutron wavelength used was $\lambda_n = 8 \text{ \AA}$, which is above the Bragg cut-off for diffraction from the crystalline lattice. The applied magnetic field was initially aligned with the incident neutron beam and parallel to the crystal's hexagonal *c* axis. Neutrons, scattered via the interaction of their magnetic moments with spatial modulations of the magnetic field, were detected on a small-angle camera positioned 17.65 m from the sample. The sum of detected images for several small inclinations ('rocking angles') of the sample and field from the incident beam direction is simply related to the Fourier transform of the magnetic field distribution in the interior of the crystal. After subtraction of background scattering observed in the normal state, the summed image thus has the approximate geometry of the real

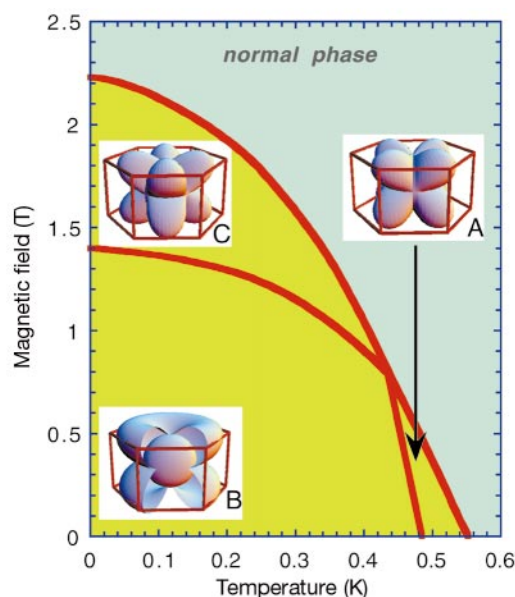


Figure 1 The low-temperature phase diagram of UPt_3 for magnetic fields parallel to the hexagonal crystal axis. Second-order phase transitions separate three different superconducting phases, labelled A, B and C. The values of the superconducting critical temperature and the critical field (at low fields) shown are the actual values measured for the crystal used in the present experiment. The geometric shapes show schematically the theoretical superconducting gap symmetries in the three phases for the E_{2u} theoretical model¹⁹. The gap magnitude is represented as the difference between the outer surfaces and inner spheres as a function of angle (a segment of the outer surface has been removed to show the inner sphere in the shape for the B phase). The frames define the hexagonal crystal symmetry. The assignment of the two shapes to the A and C phases was interchangeable depending on the sign of a coupling constant in the theoretical model: however, our experiment determines the assignment to be as shown.

FLL unit cell rotated through 90° about the field direction. But because only rocking angles about the vertical axis were explored, diffraction spots that lie almost directly above or below the incident beam are absent from the images in our study.

A schematic of the low-temperature phase diagram of UPt_3 is shown in Fig. 1. The distinctive feature of the phase diagram is the existence of three separate superconducting phases¹⁶, labelled A, B and C. The Abrikosov mixed state occurs down to very low fields, H_{c1} (not shown), of only a few thousandths of a tesla. To explain the phase diagram and other experimental results, theoretical models have attributed different symmetries to the three phases^{17,18} in accordance with group theory. The gap symmetries for one of the most successful theoretical descriptions¹⁹ (denoted E_{2u}) are shown in Fig. 1. In this model, the anisotropy of the superconducting gap in the basal plane for the A and C phases is predicted to be larger than

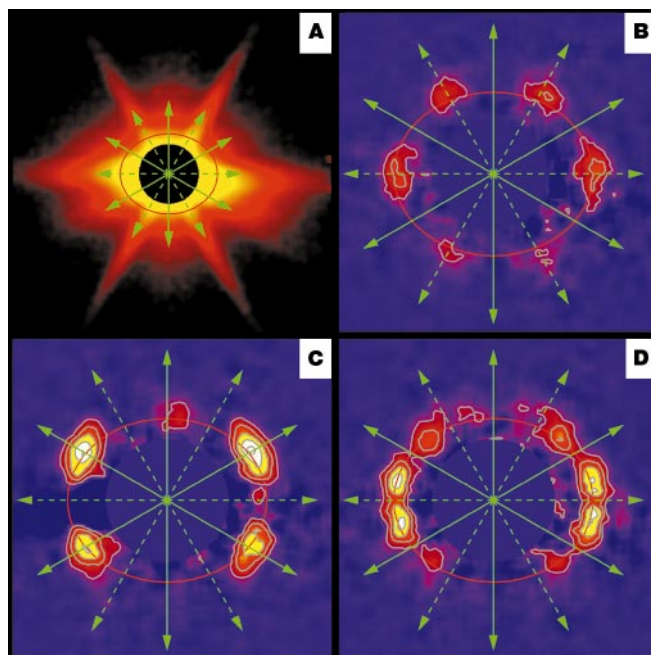


Figure 2 The measured small-angle neutron scattering from the flux-line lattice in UPt_3 . **A**, the scattering seen on the small-angle neutron camera with the sample in the normal state at 600 mK; the data are shown on a logarithmic intensity scale. The solid lines show the crystallographic *a* directions and the dashed lines the *a** directions, in the plane of the detector. The incident beam passes through the centre of the image, which is masked off. The ridges of scattered intensity parallel to crystallographic *a** directions extending towards the edges of the image have an intrinsic metallurgical origin. The subsequent panels show the extra scattering in the superconducting state at 0.19 T relative to the normal-state background. Each image is an equal combination of measurements at two rocking angles of the sample and field (-0.25° and $+0.25^\circ$ about the vertical axis). The contours are spaced in equal steps of 100 counts per pixel per hour (the detector is divided into pixels of 0.75 cm). The fact that the rocking angles differ slightly from the optimum value to illuminate each individual spot means that the centre of gravity of each diffracted spot is slightly displaced from its ideal position. Furthermore, the diffraction condition to illuminate spots lying almost vertically above or below the incident beam is not satisfied. The ellipses indicate the approximate radial positions expected for diffraction spots from an ideal hexagonal lattice with $B = 0.19 \text{ T}$ (a mean wavevector transfer of $0.0065 \text{ \AA}^{-1}$) at these rocking angles. The images all represent measurements made at low temperature (100–150 mK), but differ with respect to the field history before the measurement. Panel **B** shows the extra scattering after cooling the sample in a constant field from above T_c . Panel **C** shows the scattering after the sample was cooled in zero field, with the field applied subsequently at low temperature and then oscillated by 0.02 T about its final value. Panel **D** shows the scattering after the sample was cooled in zero field to $T_0 = 475 \text{ mK}$ (in the superconducting A phase). The field was applied and oscillated at T_0 and the sample was subsequently cooled to low temperature in a constant field.

for the B phase. The effects of both the Fermi surface anisotropy and the gap anisotropy on the orientation and symmetry of the FLL arise from non-local corrections¹¹ to the simple London model²⁰ description of the mixed state. In constant magnetic field, the non-local correction for a fixed Fermi surface anisotropy decreases only slightly (by a factor of 2) between zero temperature and the critical temperature, T_c . It acts to stabilize a hexagonal FLL aligned to the mirror planes of the hexagonal point group (along either the crystallographic $\mathbf{a}$ or $\mathbf{a}^*$ directions). The expected increase in the anisotropy of the superconducting gap with temperature close to the B–A phase line could however lead to an increasing non-local correction with a four-fold symmetry, which might be sufficient to give a measurable deviation from a perfect hexagonal FLL. The motivation for our experiment was therefore to look for possible deviations from the ideal hexagonal FLL as the A phase was approached, and additionally to test for several more exotic flux-line structures that can in theory occur in unconventional superconductors^{21,22}.

Scattering from the FLL was found to depend on the field history in the superconducting state (Fig. 2). A hexagonal FLL was always formed when the sample was cooled in a constant field from above T_c (a field-cooled history denoted as ‘FC’), with nearest-neighbour flux lines along $\mathbf{a}$ directions (Fig. 2B). The rocking width over which the diffraction spots were illuminated was measured to be rather broad (a full-width at half-maximum of 0.44°), a characteristic of a deformed FLL. The FC lattice is nucleated at the same time as the superconductivity, and is therefore sensitive to slight variations in T_c close to any defects. This gives a natural mechanism to align the FLL to the extended metallurgical structure shown in Fig. 2A. Figure 2C shows the scattering when the field was increased from zero at the measurement temperature, and then oscillated by 0.02 T about its final value (a field-oscillated history denoted as ‘FO’). The nearest-neighbour directions between the flux lines now corresponds to $\mathbf{a}^*$ directions. The rocking width was measured to be equal to the divergence of the incident neutron beam (a full-width at half-maximum of 0.14°), which is consistent with a highly ordered FLL. The same lattice orientation was in fact obtained for all field histories in which the applied magnetic field was varied significantly at low temperature, irrespective of any previous history. However, to be systematic in what follows, the crystal was

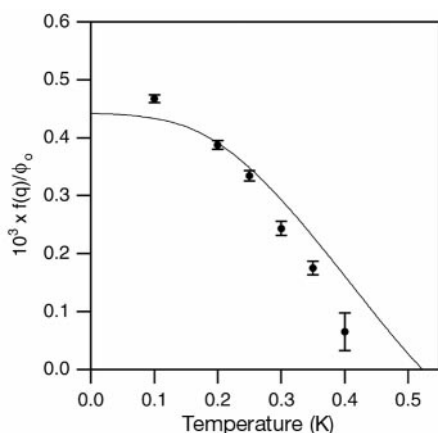


Figure 3 The measured temperature dependence of the form factor for the lowest-order diffraction process in an applied field of 0.19 T. At each temperature, the FLL was formed by the ZFC+FO process described in the text, and the diffracted intensity recorded without further changes in temperature or field. The line shows the calculated dependence of the form factor for a conventional BCS superconductor with the same parameters as our UPT₃ crystal ($dB_{c2}/dT = 7 \text{ T K}^{-1}$ at $T_c = 550 \text{ mK}$ and $\lambda(0) = 6,000 \text{ \AA}$). The observed form factor decreases more rapidly than the BCS prediction as the B–A phase transition is approached. The error bars represent the statistical errors in the diffracted intensities.

always cooled from above T_c in zero field to the temperature at which the FO procedure was applied (the full procedure is then denoted as ‘ZFC+FO’). An improvement in the degree of order of the FLL following a displacement of the flux lines, as observed here, has been reported for several conventional superconductors²³. When the pinning is not too strong, displacing the flux lines allows the lattice to relax from a frozen metastable state distorted by pinning, to approach the arrangement most energetically favoured in the absence of pinning.

Because we are interested in the equilibrium structure, we will now focus our discussion on the measurements following the ZFC+FO process, and discuss the evolution of the FLL when this process was applied at different temperatures and the FLL measured directly at these temperatures. The integrated reflected intensity for a Bragg reflection from a sample of thickness t is given by

$$R(\mathbf{q}) = \frac{\gamma^2}{16\phi_0^2 \sin(2\theta)} \frac{\lambda_0^3 t}{S_0} \frac{|f(\mathbf{q})|^2}{S_0^2} \quad (1)$$

where θ is the Bragg angle, S_0 is the unit cell area of the FLL, $\gamma = 1.91$ (the gyromagnetic ratio of the neutron), and ϕ_0 is the flux quantum. The form factor $f(\mathbf{q})$ is the Fourier component of the magnetic field distribution at a given reciprocal lattice vector $\mathbf{q} = \mathbf{G}$. In the London model, which does not consider the vortex cores, $f(\mathbf{q}) = \phi_0 / (1 + \lambda^2 q^2)$, where λ is the London penetration depth (for UPT₃ at zero temperature $\lambda = 6,000 \text{ \AA}$). A much better solution can be obtained for a one-component superconductor by numerically solving the Ginzburg–Landau theory²⁴, and can be conveniently expressed as the product of the London model solution with a correction function $g(\mathbf{q}, B/B_{c2})$, where B_{c2} is the upper critical field. However, the observed form factor for UPT₃ decreases faster with temperature

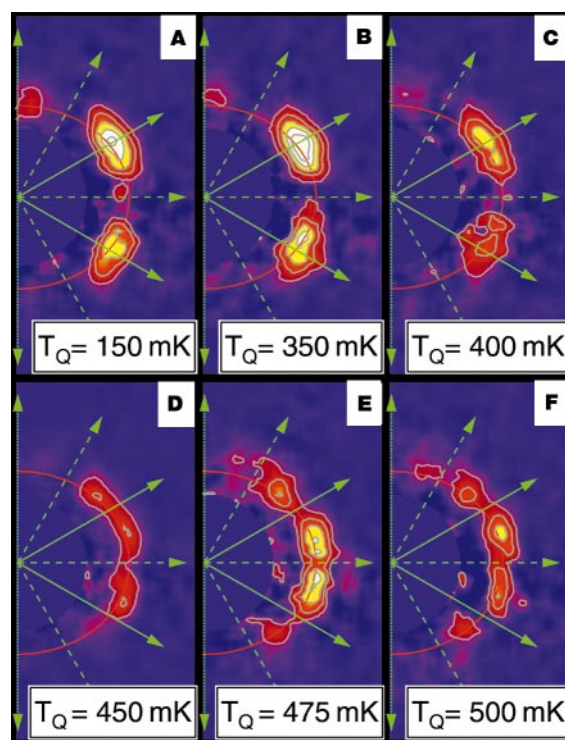


Figure 4 The transformation of the FLL orientation. The panels show the right-hand half of the detected images for diffraction from FLLs grown by the ZFC+FO procedure (see text) at various temperatures T_Q , and then quenched in constant field to low temperature (100–150 mK). The images show a transformation as the A phase is approached, from a single hexagonal lattice with nearest neighbours along $\mathbf{a}^*$ at low temperature, to two new lattice orientations rotated by -15° and $+15^\circ$ from this direction. The contours and lines have the same meaning as in Fig. 2.

than predicted by this theory for a conventional temperature-evolution of one-component Ginzburg–Landau parameters (Fig. 3). This is principally due to the presence of additional nodes in the superconducting gap, which appear as the A phase is approached. The appearance of extra nodes is consistent with Saul’s model¹⁹ and also explains the observed increase in the magnetic penetration length seen in muon measurements^{25,26}. Transverse ultrasound attenuation measurements²⁷ further show that the extra nodes lie parallel to the *c* axis. The resulting reduction in magnetic contrast, however, means that the FLL cannot be directly measured in the A phase in our experiment.

To study the A phase further, we grew FLLs at temperatures T_Q following the ZFC+FO procedure as above, but then subsequently cooled the lattices rapidly to low temperature in constant field. The quenched FLLs (Fig. 2D and Fig. 4) may now only be metastable, but they have an enhanced magnetic contrast characteristic of the lower temperature (100 mK) where the measurements were made. Following this procedure for $T_Q < 350$ mK, hexagonal lattices with the same geometry as found in the direct measurements were observed, with nearest-neighbour directions along the $\mathbf{a}^*$ axes. But for $T_Q = 450$ mK, the image appears more rotationally disordered, but weakly aligned along new directions. Remarkably, for higher T_Q ($475 \text{ mK} \leq T_Q < 520 \text{ mK}$), the observed FLLs were again extremely well ordered and clearly show this new alignment. By carefully measuring the spot positions, we conclude that for T_Q in this range the flux lines are still arranged in hexagonal lattices but with two equally populated nearest-neighbour orientations inclined at $+15^\circ$ and -15° to the crystal $\mathbf{a}$ axes.

As shown by Kogan¹¹, when $\kappa \gg 1$ ($\kappa = \lambda/\xi$, where ξ is the superconducting coherence length), the core energy of the vortices can be neglected, and the FLL symmetry can be calculated by extending the London model to include the lowest-order non-local corrections. The favoured FLL structure minimizes the free energy density, F , for a given flux density, where

$$F = \frac{B^2}{2\mu_0\phi_0} \sum_{\mathbf{q}=\mathbf{G}} f(\mathbf{q}) \quad (2)$$

The sum extends over all reciprocal lattice vectors $\mathbf{G}$, but is effectively cut off at $|\mathbf{q}| \approx 1/\xi$ by the vortex core correction to $f(\mathbf{q})$, which can be conveniently approximated²⁸ by $g(\mathbf{q}) = \exp(-\sqrt{2}q\xi)$ in the limit of $\kappa \gg 1$ ($\kappa \approx 60$ for UPt_3) and $B/B_{c2} \ll 1$. Non-local effects may additionally modify $f(\mathbf{q})$. To be more specific, we consider the angular dependence of the gap parameter in the A phase, projected into the basal plane, to be $\Delta(\mathbf{k}) \propto k_x k_y$, which is the lowest-order angular harmonic consistent with the E_{2u} model (k_x and k_y are direction cosines relative to the *x* and *y* axes). In the B phase, the second component of the order parameter is no longer zero and the basal plane gap anisotropy is smaller. This gives the following modified form for $f(\mathbf{q})$:

$$f(\mathbf{q}) = \frac{g(\mathbf{q})}{1 + \lambda^2 \mathbf{q}^2 + c_1 \lambda^4 \mathbf{q}^4 + c_2 \lambda^4 \mathbf{q}_x^2 \mathbf{q}_y^2} \quad (3)$$

where c_1 is of the order of $1/\kappa^2$. c_2 measures the degree of the basal plane anisotropy of the superconductivity. It is expected to be very small at low temperature in the B phase, but increases with temperature as the B–A transition is approached, and attains a value of the order of $1/\kappa^2$ in the A phase, where the second component of the order parameter vanishes. For $c_2 = 0$, F is a minimum for an ideal hexagonal lattice with an arbitrary orientation. For small c_2 the deformation from the hexagonal structure remains small, but the free energy is now a minimum for two lattice orientations, inclined at $+15^\circ$ and -15° to the *x*-axis. Only for $c_2 \gg 1/\kappa^2$ does the lowest-energy configuration change to approach a square lattice.

Sixth-order powers of $\mathbf{q}$ must also be included in the denominator of equation (3) to account for the anisotropy of the Fermi

surface. They are relevant only at low temperature in the B phase where the basal plane anisotropy of the superconducting gap becomes small. The crystal point group contains mirror planes along both $\mathbf{a}$ and $\mathbf{a}^*$ directions, and so, considering the Fermi surface anisotropy alone, the free energy will have extrema when the FLL symmetry also respects these symmetries; the stable FLL is then hexagonal with nearest-neighbour directions along either $\mathbf{a}$ or $\mathbf{a}^*$. It is not possible to deduce the precise anisotropy of the Fermi velocity from existing measurements of the Fermi surface, although available data²⁹ show that for the dominant sheets the projected surface areas are larger, and the electronic effective masses smaller, parallel to $\mathbf{a}$ directions. Both effects act in the same sense to suggest that the Fermi velocity, and therefore the screening currents, are larger parallel to $\mathbf{a}$. The FLL nearest neighbours would therefore be expected to lie along $\mathbf{a}^*$, as was observed for $T_Q < 350$ mK. In the theoretical description¹⁹ of the unconventional superconductivity of UPt_3 , the orientation of the order parameter and therefore of the superconducting gap is determined by the coupling of the superconductivity to a weak symmetry-breaking field. In zero magnetic field this mechanism is responsible for separating the two superconducting transition temperatures which would otherwise be degenerate. The symmetry-breaking field is usually attributed to an observed quasi-static antiferromagnetic order³⁰ with the ordered moments and ordering vectors lying along $\mathbf{a}^*$ directions³¹. In the E_{2u} model, this fixes the azimuthal orientations of the antinodes in the superconducting gap to either (1) coincide with $\mathbf{a}$ and $\mathbf{a}^*$ directions, or (2) bisect adjacent $\mathbf{a}$ and $\mathbf{a}^*$ directions; one choice occurs in the C phase, while the other is realised in the A phase (Fig. 1). From our experiment we can conclude it is the second choice that occurs in the A phase. In most other theoretical models with different candidate symmetry scenarios, such as the E_{1g} model³², the gap antinodes always lie along $\mathbf{a}$ and $\mathbf{a}^*$ directions and they cannot therefore explain the unusual FLL orientation we observe. It is important also to stress that our result is independent of any domain structure of the symmetry-breaking field. Different domains will be related by rotations through multiples of 60° , but give rise to the same hexagonal FLL. $\square$

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Shaped-pulse optimization of coherent emission of high-harmonic soft X-rays

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When an intense laser pulse is focused into a gas, the light–atom interaction that occurs as atoms are ionized results in an extremely nonlinear optical process^{1–3}—the generation of high harmonics of the driving laser frequency. Harmonics that extend up to orders of about 300 have been reported^{4,5}, some corresponding to photon energies in excess of 500 eV. Because this technique is simple to implement and generates coherent, laser-like, soft X-ray beams, it is currently being developed for applications in science and technology; these include probing the dynamics in chemical and materials systems⁶ and imaging⁷. Here we report that by carefully tailoring the shape⁸ of intense light pulses, we can control^{9,10} the interaction of light with an atom during ionization, improving the efficiency of X-ray generation by an order of magnitude. We demonstrate that it is possible to tune the spectral characteristics of the emitted radiation, and to steer the interaction between different orders of nonlinear processes.

This work builds on a number of other recent advances in laser and nonlinear-optical science and technology. The use of temporally shaped ultrashort pulses to create ‘designer’ atomic wavepackets¹¹, to control two-photon absorption^{12,13}, and to control molecular processes^{11,14} has been a topic of increasing interest as laser technology has developed. Recent work has demonstrated techniques to increase the conversion efficiency of high-harmonic generation by using waveguide¹⁵ and new focusing geometries^{16,17}, and very short duration (≤ 25 fs, or ~ 10 optical cycles) light pulses¹⁸. It has also been shown that the temporal ordering of

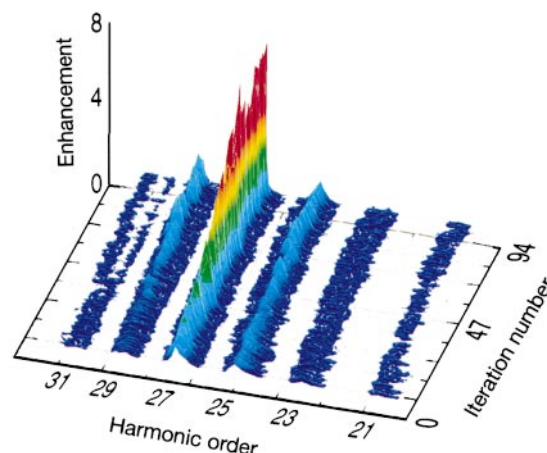


Figure 1 Optimization of a single (27th) harmonic in argon while suppressing adjacent harmonics. The optimization criterion (fitness function) for this run corresponded to $f = \Sigma s_j - \Sigma s_i - \Sigma s_k$, where s corresponds to the signal level of a CCD pixel, and the ranges i , j and k represent the pixels corresponding to a 0.5-nm spectral bandwidth centred around the 25th, 27th and 29th harmonic. The peak enhancement for the 27th harmonic is a factor of 8, while the energy enhancement is a factor of 4.6. The contrast ratio between the 27th harmonic and adjacent harmonic increases by a factor of 4.

colours in the light pulse affects the spectral characteristics of the high-harmonic radiation^{19,20}. However, that work was limited to simple adjustment of the light pulse ‘chirp’ and did not reveal any ability to selectively control the high harmonic generation (HHG) process or to improve overall conversion efficiency.

The present work uses a newly developed laser system that generates high-power ultrafast laser pulses with the capability of temporally reshaping them in a very precise and flexible manner²¹. We show that very subtle changes (resulting in changes in the time duration of the light pulse of only a few femtoseconds) can manipulate the electronic response of an atom, and thereby control the spectral characteristics and brightness of the soft X-ray coherent radiation. We have found this effect to be very general, occurring over a wide range of parameters and in different gases. In light of past work, the effectiveness of this technique is unexpected. It illustrates significant differences between low-order nonlinear processes such as second-harmonic generation and high-order processes such as HHG that are inherently non-perturbative. Furthermore, under some conditions it is possible to channel most of the high-harmonic emission into a single spectral peak creating a coherent, nearly monochromatic soft X-ray source with a greatly enhanced brightness.

In our experiment, we focus light pulses from an ultrafast laser system with a repetition rate of 1 kHz and a pulse energy of up to 1 mJ into a 175- μm diameter, gas-filled capillary waveguide. The pulse peak intensity ($\sim 2 \times 10^{14} \text{ W cm}^{-2}$) is sufficient to field-ionize the atoms (typically argon), in the process generating high-harmonic extreme ultraviolet (XUV) radiation. The capillary waveguide allows us to create an extended region of high laser intensity and long coherence length to efficiently generate the high-harmonic radiation¹⁵, which is observed using an X-ray charge-coupled device (CCD) camera directly coupled to a grazing-incidence spectrometer.

The laser system incorporates a micromachined deformable mirror pulse-shaping apparatus²². This adaptive optic element is used in a position within the laser system where the frequency components of the light pulse are spatially separated and can change the relative path length of the various colours, thus reordering the arrival times of the colours of the short pulse. The light pulse bandwidth is about 80 nm full-width at half-maximum (FWHM) centred at 800 nm, and is not altered by the pulse shaper.

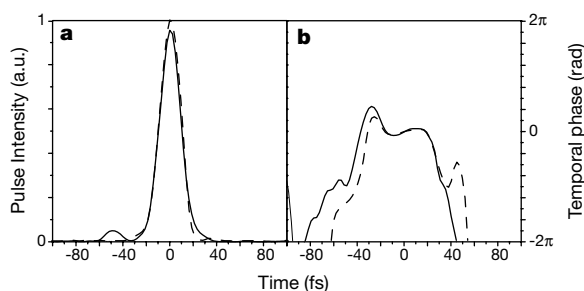


Figure 2 Laser pulse characteristics. **a**, **b**, Amplitude (**a**) and phase (**b**) of the transform-limited (dashed line) and optimized (solid line) laser pulses corresponding to Fig. 1. These data were taken using the frequency-resolved optical gating (FROG). The energy of the pulse is kept constant.

We have shown that this mirror, when combined with adaptive control using an evolutionary search procedure, can allow us to generate nearly perfectly transform-limited, 15-fs (<6 optical cycles) light pulses from the laser²¹. To do so, we optimized the intensity of second-harmonic radiation (SHG) generated by the laser pulses passing through a thin frequency-doubling crystal²³. The actual laser pulse shapes were characterized in both amplitude and phase using the frequency-resolved optical gating (FROG) technique²⁴. In the case of SHG-optimized control, the highest conversion efficiency was observed to correspond to the highest peak power obtainable for a given pulse spectrum (that is, the time-bandwidth or Fourier-transform limit of laser pulse, where all frequency components arrive at once). This is the intuitive result, since a nonlinear-optical process should be most efficient at the highest peak intensity.

For our experiments to optimize HHG, we started with an 18-fs, Fourier-transform-limited laser pulse obtained using the SHG optimization technique. The initial curve of Fig. 1 (iteration number 0) shows the HHG spectrum from argon gas at about the 27th harmonic, using a transform-limited 18-fs pulse. This curve also represents the highest overall signal level obtainable before learning optimization of the HHG. We verified that adjustment of the linear chirp of the pulse did not improve the brightness of the HHG spectrum. In order to use the evolutionary strategy (ES) for HHG optimization, a spectral window of 0.5 nm surrounding the 27th harmonic was selected.

The algorithm starts with a set of randomly selected pulse shapes (corresponding to 19 random voltages on the pulse shaper), and searches for a pulse shape to maximize the signal intensity here while at the same time minimizing the adjacent 25th and 29th order harmonics. The best (fittest) pulses are then randomly altered (mutated), by adding a normally distributed random variable to the value of each pixel, and retested. Each 'parent' solution generates five mutated 'children'. The algorithm converges to some optimal solution after approximately 50 iterations. Figure 1 shows the optimum solution after each iteration of the algorithm.

Surprisingly, after optimization the intensity of a single harmonic (27th) has been increased by a factor of eight, while adjacent harmonic orders are only slightly enhanced. The level of enhancement depends on the gas pressure, harmonic order and gas type, with the largest enhancements (of over an order of magnitude) observed at a gas pressure corresponding to macroscopic phase-matching of the high-harmonic process, and for the harmonic (27th) where neutral gas absorption of the light is near the minimum. However, enhancement of more than three times is observed under virtually every set of conditions. As different optimization searches proceed, the relative heights of the various harmonics can change, and energy is channelled to the 27th harmonic as the search converges. The total integrated X-ray flux also increases by a factor of two to six, depending on the gas pressure and harmonic orders

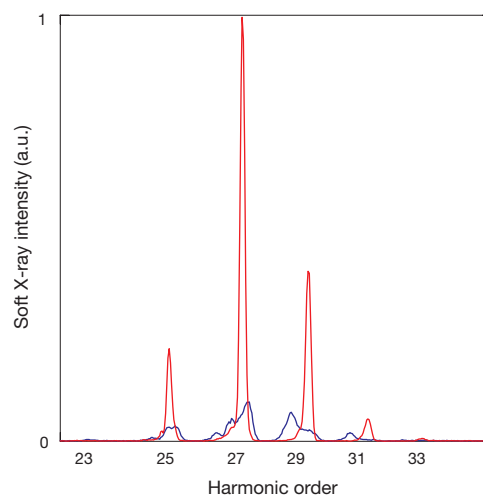


Figure 3 Optimization of a single harmonic in argon. The spectral window occurs at longer wavelengths than in Fig. 1 and without suppressing adjacent harmonics. That is, the fitness function is $f = \sum S_j$, where the range j represents pixels to the 'red' side of the 27th harmonic. The harmonic peak is enhanced by over an order of magnitude. Harmonics before and after optimization are shown in blue and red respectively.

being optimized. For example, in the case of krypton gas, the combined nonlinearity and absorption characteristics of the gas result in about six harmonic peaks of comparable intensity before optimization. Optimization of any particular peak in the range of 17–23 results in enhancement of this peak by a factor of three to four times, while harmonics immediately adjacent to the 'target' peak are enhanced by about 1.5–2 times, and other peaks have negligible or negative enhancement.

Figure 2 shows the laser pulse shapes corresponding to the transform-limited and final HHG spectra shown in Fig. 1. The transform-limited and optimized laser pulse shape are shown in Fig. 2a, while Fig. 2b shows the corresponding phase values. The optimized pulse has more structure and phase change on the leading edge, but is not much broader than the transform limit (~ 21.6 fs compared with 18 fs). Thus, very slight modification of the driving pulse can result in drastic changes in the harmonic radiation generated.

We have found that different optimization runs, using the same fitness criteria, yield slightly differing pulse shapes even when similar enhancements are observed, suggesting that some degrees of freedom for the pulse shape are not significant, while others are; work is in progress to modify the fitness criteria to try to identify the critical features. The enhanced pre-pulse observed in the optimized pulse is not always present, and is probably a result of limitations of the phase-only pulse shaper; however, the presence of some phase structure on the leading edge of the pulse is a consistent feature. Furthermore, as the intensity of the pre-pulse is below that required for ionization and therefore HHG emission, it is unlikely to be important. By selecting different criteria (that is, different spectral windows, different harmonics, and/or different gases), the ES search algorithm can converge on different optimal solutions. Figure 3, for example, shows the results before (blue) and after (red) optimization of the 27th harmonic in argon, but in this case optimized over a shifted spectral window positioned to the left of the harmonic peak, with no suppression of adjacent peaks.

The optimization process increased both the peak intensity and spectral purity of the harmonic, increasing the peak by over an order of magnitude. Choice of the optimization window on the red or the blue side of the initial peak consistently results in a red or blue shift of the harmonic; however, the resulting spectral bandwidth varies. This optimization is consistent with previous work that showed that

a positive (red to blue) chirp on the driving pulse can result in both a red shift and a spectral narrowing of the harmonic emission peaks¹⁹ (although in that case no dramatic increase in brightness or selectivity was observed). The measured width of the optimized 27th harmonic is 0.24 eV, corresponding to our instrument resolution. Thus, the true peak and spectral enhancements may be even higher. Since the duration of the XUV pulse is probably somewhat shorter than the driving laser pulse, the resulting HHG spectrum corresponds to an XUV pulse much closer to the time-bandwidth limit than before optimization. This result illustrates quite clearly that pulse shaping can alleviate what had been thought to be a fundamental 'trade-off' for use of HHG as a light source—that the use of very short driving pulses, although dramatically increasing the efficiency of HHG, results in a broader spectrum for the individual harmonic peaks.

To explain the unexpected result that very slight changes in laser pulse shape can dramatically enhance and select individual harmonics, we consider the semi-classical rescattering model of HHG^{25,26}. From a classical point of view, electrons are ionized and accelerated away from the core during one half-cycle of the laser field, and can be subsequently driven back to the core when the laser field reverses. Some fraction of the ionized electrons can recombine with the parent ion and give off their energy in the form of high harmonics. This process occurs over a few optical cycles of the laser field, resulting in an approximately 5-fs X-ray burst. Particular harmonics are generated by electrons returning to the core with a specific return energy that is related to the exact time within the optical cycles when the electron is initially ionized.

We believe that the adaptive optimization algorithm finds a pulse with the correct phase sequence to ensure that the continuum X-ray emission generated by a particular cycle of the laser pulse reinforces constructively or destructively with different parts of the continuum generated by adjacent cycles. This coherent control can lead to channelling and redirection of energy between different high-order nonlinear interactions.

These findings therefore demonstrate a new type of intra-atomic 'phase matching' between the laser field and the wavefunction of the ionized electron. Preliminary calculations based on phase-only pulse shaping confirm this interpretation. From a quantum point of view, our optimized laser pulse can adjust the quantum phase of the electron wavefunction which returns to the core, to optimize it for a particular harmonic feature. This work may lead to other new methods for control of highly nonlinear systems, as well as improving the utility of the HHG source for application experiments in a broad range of science^{27–30}. □

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Configurational entropy and diffusivity of supercooled water

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As a liquid approaches the glass transition, its properties are dominated by local potential minima^{1,2} in its energy landscape. The liquid experiences localized vibrations in the basins of attraction surrounding the minima, and rearranges via relatively infrequent inter-basin jumps³. As a result, the liquid dynamics at low temperature are related to the system's exploration of its own configuration space. The 'thermodynamic approach' to the glass transition considers the reduction in configuration space^{4–8} explored as the system cools, and predicts that the configurational entropy^{5,9,10} (a measure of the number of local potential energy minima sampled by the liquid) is related to the diffusion constant. Here we report a stringent test of the thermodynamic approach for liquid water (a convenient system to study because of an anomalous pressure dependence in the diffusion constant). We

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calculate the configurational entropy at points spanning a large region of the temperature–density plane, using a model¹¹ that reproduces the dynamical anomalies of liquid water. We find that the thermodynamic approach can be used to understand the characteristic dynamic anomalies, and that the diffusive dynamics are governed by the configurational entropy. Our results indicate that the thermodynamic approach might be extended to predict the dynamical behaviour of supercooled liquids in general.

One of the most studied models of molecular liquids is the extended simple point charge (SPC/E) potential, designed to model the behaviour of water¹¹. The dynamic properties of this model have been studied in detail in the weakly supercooled regime where the diffusion constant decreases by three or four orders of magnitude compared to its normal liquid value ($\sim 10^{-5} \text{ cm}^2 \text{ s}^{-1}$). In this temperature range the SPC/E dynamics have been shown to be consistent^{12,13} with the predictions of the mode-coupling theory (MCT)^{14,15}, and the locus of critical MCT temperatures $T_{\text{MCT}}(\rho)$ is also known¹³. The SPC/E potential is of particular interest for testing theories of the supercooled-liquid dynamics because, as observed experimentally for water, the diffusion constant D has a maximum as a function of the pressure P —or of the density ρ —along isotherms, and this maximum becomes more pronounced upon cooling¹⁶.

Here we calculate the SPC/E liquid entropy S , the vibrational entropy S_{vib} of the liquid constrained in one typical basin of the potential energy landscape, and the configurational entropy S_{conf} defined by $S_{\text{conf}} \equiv S - S_{\text{vib}}$, for state points covering a large region of the (T, ρ) phase diagram. Figure 1 shows the calculated entropies for one particular density. Figure 2a–c shows S , S_{vib} and S_{conf} as functions of ρ for several isotherms. Figure 2d shows the behaviour of D along the same isotherms. Both S_{conf} and D show maxima which become more pronounced with decreasing temperature. Figure 2c and d demonstrates a remarkable correlation between the qualitative behaviours of S_{conf} and D .

Figure 2 also shows that at all temperatures, both D and S_{conf} have maxima at $\rho \approx 1.15 \text{ g cm}^{-3}$. The presence of a maximum in S_{conf} at $\rho \approx 1.15 \text{ g cm}^{-3}$ is possibly explained by two density-dependent (and nearly temperature-independent) mechanisms balancing. Increasing density in water from the ‘ideal’ tetrahedral density (for ice, about 0.92 g cm^{-3}) leads to the progressive destruction of the hydrogen-bond network. Hence the number of potential energy minima increases (and therefore so does S_{conf} which is a measure

of the number of local landscape minima), as there are more configurations corresponding to a disordered tetrahedral network. At large enough density, core repulsion dominates the liquid properties, and we indeed find that increasing density decreases S_{conf} (as fewer configurations are possible when the system becomes more densely packed).

The close connection between S_{conf} and D shown in Fig. 2 occurs in the same region of the (T, ρ) plane where the dynamics of SPC/E water can be rather well described by MCT^{12,13}. Hence MCT appears to capture the reduction of the mobility due to entropic effects.

The phase diagram of liquid water is characterized by the well-known isobaric density maxima line as well as a line of isothermal D maxima. The density maximum line coincides, from the Maxwell relation, $(\partial V/\partial T)_P = -(\partial S/\partial P)_T$, to a line of S extrema. The lines of maximum S and maximum D in real water (Fig. 3a) and in SPC/E simulated water (Fig. 3b), do not coincide. The hypothesis that S_{conf} —not S —is the relevant quantity controlling the dynamics in supercooled states can be tested by comparing the line of isothermal S_{conf} maxima with the line of D maxima. Figure 3b shows that the lines of maxima for S_{conf} and D coincide within calculation uncertainties.

While the thermodynamic approach does not provide a quantitative prediction for D , our calculations allow us to test the functional form relating D and S_{conf} proposed by Adam and Gibbs⁵. In the range of D values that we have probed (Fig. 3c), we find agreement with the linear relation between $\log D$ and $(TS_{\text{conf}})^{-1}$ proposed theoretically⁵ and verified experimentally in a smaller D range¹⁷.

Our results for S_{conf} permit us to determine the locus of $T_K(\rho)$, which is calculated as the temperature¹⁸ where $S_{\text{conf}} \rightarrow 0$. Note that Kauzmann defined T_K to be the temperature at which the liquid entropy becomes equal to the crystalline entropy along an isobaric path. Kauzmann’s definition coincides with the definition used here if the crystal vibrational entropy is a good approximation to the basin vibrational entropy. Our definition of $T_K(\rho)$ is related to

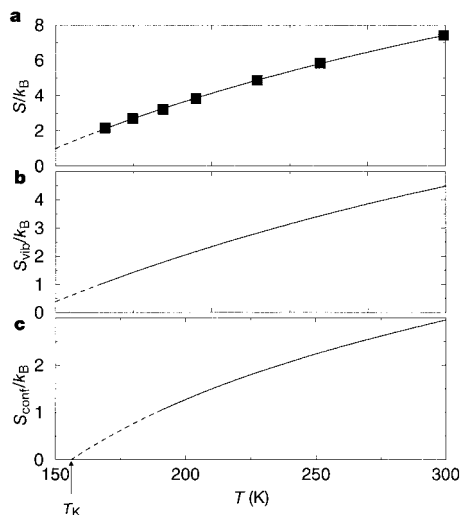


Figure 1 Calculated values of the total vibrational and configurational entropy for one particular isochore, $\rho = 1.0 \text{ g cm}^{-3}$. **a**, S ; **b**, S_{vib} ; **c**, S_{conf} . The arrow indicates the Kauzmann temperature T_K where $S_{\text{conf}} \rightarrow 0$.

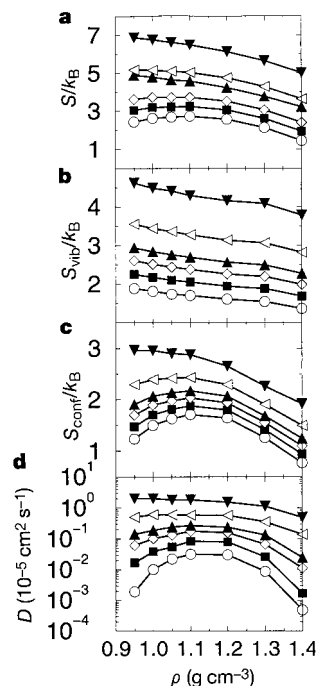


Figure 2 Density dependence of four quantities. **a**, S ; **b**, S_{vib} ; **c**, S_{conf} ; and **d**, D (data from ref. 13), shown along six constant-temperature paths; top to bottom $T = 300$, $T = 260 \text{ K}$, $T = 240 \text{ K}$, $T = 230 \text{ K}$, $T = 220 \text{ K}$, $T = 210 \text{ K}$. We note the close correspondence of densities where the maxima of S_{conf} and D occur.

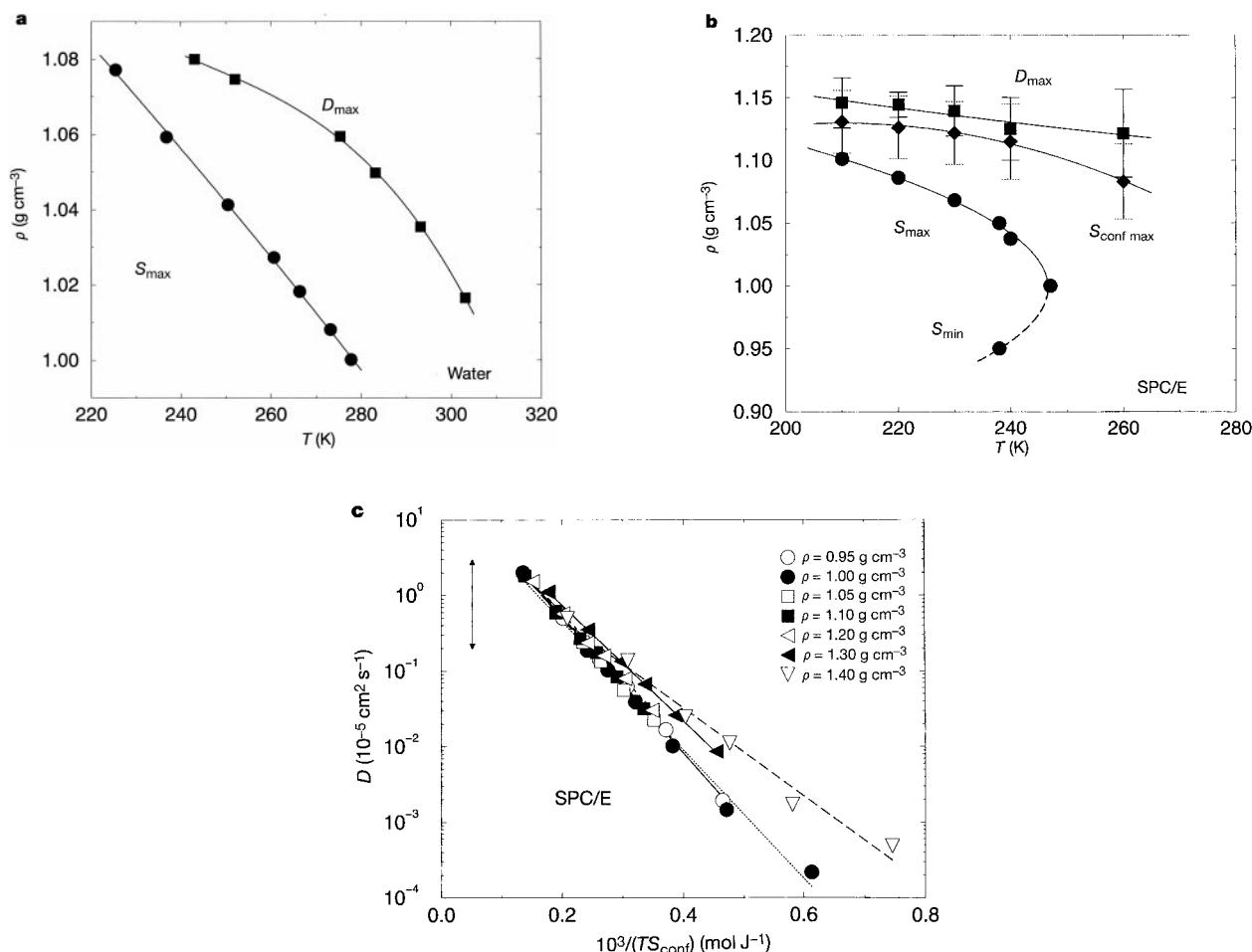


Figure 3 Lines of entropy and diffusion constant extrema, comparing experiments and the present simulations. **a**, Lines of entropy maxima and diffusion constant maxima taken from experimental data on water¹⁶. **b**, Lines of entropy extrema, configurational entropy maxima, and diffusion constant maxima D for the SPC/E model. Note that the line of

entropy maxima does not coincide with the line of diffusion constant maxima. **c**, Semi-log plot of the diffusion constant D versus $(TS_{\text{conf}})^{-1}$ for six isochores. The double arrow denotes the range of D values where the relationship $\log D$ versus $(TS_{\text{conf}})^{-1}$ has been experimentally tested in bulk water¹⁷. The lines are provided as a guide to the eye.

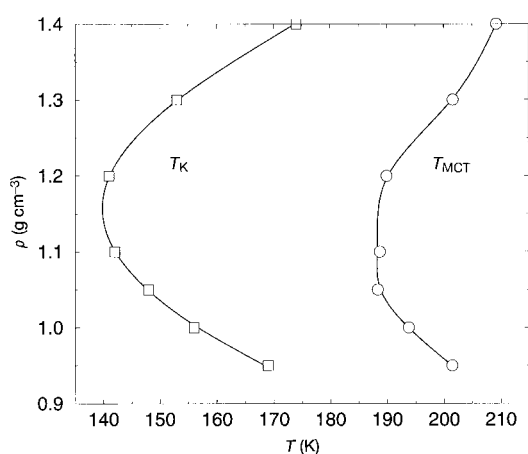


Figure 4 The locus of the mode-coupling theory transition temperature T_{MCT} (ref. 13) and the locus of the Kauzmann temperature T_K . The locus $T_K(\rho)$ is estimated by performing an extrapolation of the (T, ρ) dependence of the configurational entropy. In the context of estimating T_K from the analysis of experimental or numerical data, no distinction can be made between a scenario where S_{conf} indeed goes to zero at T_K (with a clear break in the first T derivative) and a scenario where the extrapolated S_{conf} has a dramatic change in slope and approaches zero only at a much lower T . A slower change in S_{conf} is expected to be accompanied by a slower decrease of D , related to a possible fragile-to-strong transition in water^{27–29}, a topic of current debate.

the original definition of the Kauzmann temperature because at $T_K(\rho)$, the liquid S_{conf} reaches the crystalline S_{conf} , and thus at $T_K(\rho)$ the number of basins populated in thermal equilibrium is non-extensive. To estimate the difference in the two definitions, we also calculate the purely vibrational ice I_h crystal entropy, and we find that the value for $T_K(\rho)$ is roughly 5 K lower than the one shown in Fig. 4. The locus $T_K(P)$ can be estimated from knowledge of $T_K(\rho)$ and from the equation of state using the implicit relation for the pressure, $P = P(\rho, T_K(\rho))$.

The region $T < T_{\text{MCT}}$ defines the region where basin changes require a basin-hopping mechanism¹⁹. The ratio T_{MCT}/T_K is one measure of the fragility of a liquid²⁰, and $T_{\text{MCT}}/T_K \rightarrow 1$ is expected for an ideal fragile liquid. Figure 4 shows both T_K and T_{MCT} ; we see that $T_{\text{MCT}}/T_K \approx 1.20$ – 1.35 , indicating that the liquid modelled by the SPC/E potential is fragile in the region of temperatures and densities studied here.

Finally, we note that our approach provides us with an estimate of the free energy which can be extrapolated to low temperature to search for the hypothesized liquid–liquid critical temperature^{21,22}. We find that, for the SPC/E potential, the critical point lies below the locus of $T_K(\rho)$ and hence cannot be accessed in equilibrium. □

Methods

Evaluation of $S(T, \rho)$

We first calculate S for a reference point ($T = 1,000$ K and $\rho = 1.0$ g cm⁻³), and then calculate the entropy as a function of (T, ρ) via thermodynamic integration using the state

points simulated in ref. 13, and new simulations extending to higher temperatures. Specifically, we integrate the relationship $dS = dE/T + PdV/T$ along both constant T and constant V paths. To evaluate S ($T = 1,000$ K, $\rho = 1.00$ g cm $^{-3}$), we perform thermodynamic integration along the $T = 1,000$ K isotherm starting from the known analytic value for an ideal gas of rigid triatomic molecules in the infinite volume limit. We verify the reliability of our results for S by checking that $\phi dS = 0$ along several closed P – V cycles. It is convenient to find a functional form to fit the data for $S(T)$. Recently it has been proposed theoretically²³ that—in classical fluids—the temperature dependence of the potential energy at constant volume follows a $T^{3/5}$ law on cooling, contributing an entropy variation that follows a $T^{-2/5}$ law. This temperature dependence has been verified in several atomic system, such as Lennard–Jones, soft spheres, and charged systems in the limit of large densities or low T (ref. 23). We find that temperature dependence is numerically obeyed in our molecular system for $200 < T < 300$, and we use it to generate an analytic expansion for the free energy and the solid line in Fig. 1a.

Calculation of S_{vib}

We use the technique presented in refs 24 and 25 to study the properties of the basins visited by the liquid at equilibrium. For each (T, ρ) we use the conjugate gradient algorithm²⁵ to calculate—for two independent systems and for 100 configurations—the corresponding local minima, called inherent structures². The configurations we use span a time of at least 25 times the α -relaxation time of the density–density correlation function. We estimate S_{vib} by adding anharmonic corrections to the harmonic contribution $S_{\text{harm}} = k_B \sum_{i=1}^{3N-6} [\ln(k_B T \hbar \omega_i) - 1]$, where ω_i are the normal-mode frequencies of the inherent structure determined from the Hessian matrix¹⁹. Here k_B is Boltzmann's constant, N is the number of molecules and $\hbar$ is Planck's constant. The harmonic approximation for a binary Lennard–Jones mixture is a valid estimate of S_{vib} for temperatures around T_{MCT} (refs 25, 26). However, in the case of the SPC/E potential, we find that there are significant anharmonicities in the basins. This can be seen from the fact that, if the system were purely harmonic, the energy E should equal E_{basin} , the energy of a minimum, plus the contribution $E_{\text{harm}} = (6N - 3)k_B T/2$ of the harmonic solid approximation. In contrast with the binary mixture Lennard–Jones case^{25,26}, we find the vibrational contribution $E_{\text{vib}} \equiv E - E_{\text{basin}}$ to be roughly 10% larger than the harmonic approximation, even at the lowest temperatures studied.

We estimate therefore the anharmonic contributions to S_{vib} by heating inherent structures at constant volume and measuring the deviation of E_{vib} from the harmonic approximation. For each collection of basins corresponding to a particular (T, ρ) state point, we calculate E_{vib} in the range $T = 0$ – 200 K and fit our results using the approximation $E_{\text{vib}} = E_{\text{harm}} + aT^2 + bT^3$ with a, b used as fitting parameters. By integrating the relation $dS_{\text{vib}} = dE_{\text{vib}}/T$, we find $S_{\text{vib}} = S_{\text{harm}} + 2aT + \frac{3}{2}bT^2$. We have checked that system remains confined in the same basin up to $T \approx 200$ K when heating the inherent structure from $T = 0$ K on the time scale of 200 ps. We have evaluated this anharmonic contribution from the inherent structures of the simulations at $T = 210$ K. However, using the inherent structures generated from higher temperature configurations does not affect our results.

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Electronic connection to the interior of a mesoporous insulator with nanowires of crystalline RuO₂

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Highly porous materials such as mesoporous oxides are of technological interest¹ for catalytic, sensing and remediation applications: the mesopores (of size 2–50 nm) permit ingress by molecules and guests that are physically excluded from microporous materials. Connecting the interior of porous materials with a nanoscale or ‘molecular’ wire would allow the direct electronic control (and monitoring) of chemical reactions and the creation of nanostructures for high-density electronic materials². The challenge is to create an electronic pathway (that is, a wire) within a mesoporous platform without greatly occluding its free volume and reactive surface area³. Here we report the synthesis of an electronically conductive mesoporous composite—by the cryogenic decomposition of RuO₄—on the nanoscale network of a partially densified silica aerogel. The composite consists of a three-dimensional web of interconnected (~ 4 -nm in diameter) crystallites of RuO₂, supported conformally on the nanoscopic silica network. The resulting monolithic (RuO₂||SiO₂) composite retains the free volume of the aerogel and exhibits pure electronic conductivity. In addition to acting as a wired mesoporous platform, the RuO₂-wired silica aerogel behaves as a porous catalytic electrode for the oxidation of chloride to molecular chlorine.

The metallic electronic conductivity of anhydrous RuO₂ (single-crystal conductivity, σ , $\sim 2 \times 10^4$ S cm $^{-1}$ at 25°C), along with its excellent chemical and thermal stability^{4–7}, makes this material feasible as a durable nanowire in the presence of water and oxygen,

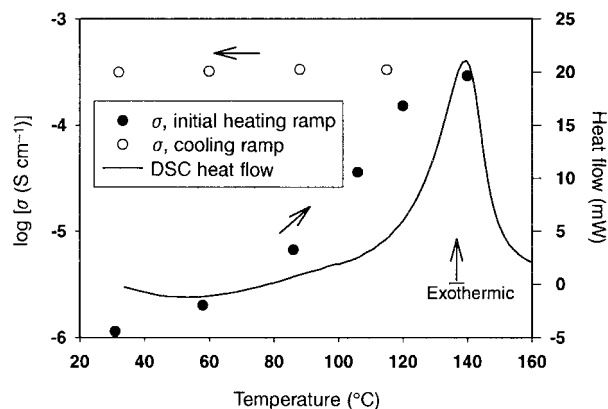


Figure 1 Impedance measurements on a RuO₂-coated SiO₂ aerogel. The sample was heated at 2 °C min⁻¹ in flowing O₂, held at 150 °C for 1 h, and then cooled to room temperature at 2 °C min⁻¹; the conductivity, σ , is based on the geometric area of the composite (6.4-mm diameter, 1.1-mm thick disk) and does not reflect the innate conductivity of the RuO₂ nanowire. Superimposed on the σ - T plots is the differential scanning calorimetric heat flow curve for a RuO₂/SiO₂ aerogel composite heated at 2 °C min⁻¹ under O₂.

unlike metal-based nanowires⁸. Silica aerogels are mesoporous platforms in which mass transport occurs within a continuous three-dimensionally mesoporous network⁹ and is not restricted to two-dimensional flux through channels, as is typical of almost all template-synthesized mesoporous materials¹. The high surface area (up to 1,000 m² g⁻¹) and continuous porosity (up to more than 99%) of aerogels are generally achieved by extracting the solvent from the pores of a wet gel under supercritical conditions, thereby avoiding shrinkage caused by capillary forces that develop during ambient drying⁹. Silica aerogels can be prepared as robust monoliths with extremely low electrical and thermal conductivity^{9,10}.

Conventional RuO₂-based electrodes, used for the chloralkali process or as thick-film resistors, are usually prepared by thermal oxidation⁷ or chemical vapour deposition (CVD)¹¹ of appropriate Ru-based precursors. Similar techniques have been used to perfuse secondary phases into aerogel pores¹²⁻¹⁴. We have previously

used both vapour and liquid infiltration methods, with Ru organometallic precursors such as Ru(III)tris-acetylacetonate and ruthenocene, to produce RuO₂/SiO₂ aerogel composites^{13,14}. These composites were not conductive, however, because the decomposition temperatures necessary to form RuO₂ volatilized much of the precursor out of the aerogel structure and coarsened the RuO₂ that did deposit, thereby separating neighbouring particles.

To solve this problem, we developed a low-temperature, solution-infiltration approach that synthesizes a conductive path through SiO₂ aerogels by cryogenic decomposition of an alternative precursor, RuO₄. Ruthenium tetroxide has been used as a non-cryogenic precursor to deposit RuO₂ films at 150 °C on smooth substrates¹⁵, decorate biological specimens with conductive RuO₂ particles in order to improve micrographic contrast¹⁶, and synthesize conductive RuO₂ films on polymer substrates from RuO₄/pentane solutions (ref. 17 and references therein). When the temperature of a RuO₄/pentane solution is raised slowly from -78 °C after equilibration with the SiO₂ aerogel substrate, RuO₂ preferentially forms on the aerogel surfaces rather than precipitating as powdered RuO₂. These composites are electronically conductive throughout, on the basis of two-point probe conductivity tests between various points in the centre and on the outer surfaces of the aerogel.

The impedance of bulk RuO₂/SiO₂ aerogel composites exhibits only a resistive component from 0.1–1 MHz. The as-deposited conductivity is modified significantly by annealing. Heating the nanocomposite under flowing oxygen to about 150 °C increases the conductivity by 2–3 orders of magnitude (Fig. 1) to about 1 mS cm⁻¹, but additional heating to temperatures greater than about 230 °C produces a decrease in conductivity to levels at or below the as-deposited values.

The increase in conductivity upon heating the composite to 150 °C is maintained upon cooling to room temperature (Fig. 1). The thermopower measured for these annealed samples is consistent with metallic behaviour (linear in voltage versus temperature) with a Seebeck coefficient of 3.9–4.4 μ V K⁻¹. The thermoelectric character of sputtered thin film RuO₂ has previously been studied and a coefficient of 4 μ V °C⁻¹ was obtained¹⁸. Without an oxygen anneal, the material is no longer conductive after a period of several days in ambient laboratory conditions. Heating under a low partial pressure of oxygen (flowing argon) results in a complete loss of

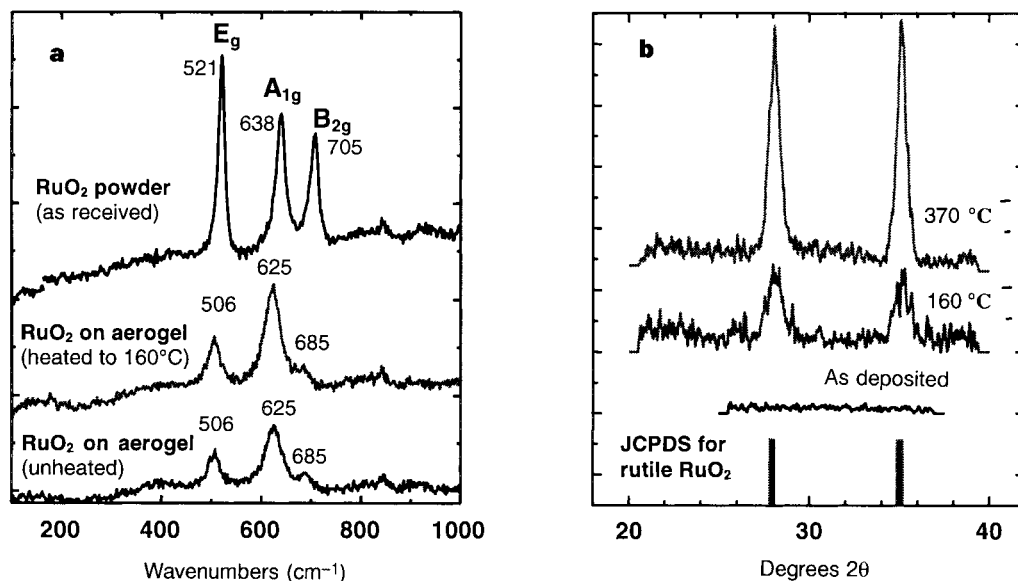


Figure 2 Comparison of nanoscale RuO₂ and polycrystalline, anhydrous RuO₂. Nanoscale RuO₂ was deposited on partially densified SiO₂ aerogel by the cryogenic decomposition of

RuO₄. **a**, microprobe Raman spectroscopy; **b**, X-ray diffraction.

electrical transport at 250 °C; X-ray photoelectron spectroscopic analysis of these composites indicates that deoxygenation of the nanoscale RuO₂ occurred. An oxygen-annealed RuO₂||SiO₂ aerogel composite that had been immersed in water for months retained comparable conductivity to its initial value after emerging and air-drying.

The cryogenically formed RuO₂, whether deposited on the internal surfaces of the aerogel or precipitated as a separate powder, is amorphous on the basis of X-ray diffraction. On the molecular scale, which was probed by Raman spectroscopy, both forms are structurally related to polycrystalline RuO₂ (ref. 19) and they exhibit identical Raman spectra (see Fig. 2a for the spectrum of the aerogel-supported, cryogenically formed RuO₂). The 15–20 cm⁻¹ red-shifts in peak position and the different intensity ratios seen for the cryogenically obtained oxide relative to polycrystalline anhydrous RuO₂ are attributed to the nanoscale nature of the oxide. Red-shifts in the Raman peak positions for RuO₂ thin films relative to those of single-crystalline RuO₂ are known, with the peaks broadening as the films become less well ordered²⁰.

An exothermic peak in the differential scanning calorimetry data (Fig. 1) indicates that the aerogel-supported, oxygen-annealed nanoscale particles of RuO₂ crystallize at about 120 °C with a heat of crystallization of -78 ± 16 kJ mol⁻¹. Samples heated to 160 °C exhibit X-ray diffraction peaks corresponding to rutile RuO₂ (see Fig. 2b). Heating to higher temperatures under oxygen leads to crystallite coarsening and narrowing of the X-ray diffraction peaks. However, a concomitant drop in electronic conductivity occurs, which implies that such coarsening leads to physical breaks in the wiring that makes up the electronic pathway through the RuO₂ nanoweb.

The morphology and crystal structure of the as-deposited and oxygen-annealed RuO₂ network within the porous aerogel were characterized using transmission electron microscopy (TEM). Before annealing, the deposited RuO₂ has electron diffraction patterns that appear amorphous, however, a few weak lattice fringes

are observed, which indicate some local crystallinity. The as-deposited RuO₂ particles form a conformal layer about 2 nm thick over a portion of the aerogel surfaces (Fig. 3a) that self-wires the electron path through the aerogel interior. The side-by-side assembly (and accompanying electron connectivity) of the spheres of RuO₂ on the SiO₂ network can be visually traced from the outer edges of the aerogel, which explains why the as-synthesized composite exhibits electronic conductivity.

Heating the as-deposited composite to 145 °C under oxygen, which improves the bulk conductivity by 2–3 orders of magnitude (see Fig. 1), forms 3–5-nm RuO₂ crystals (Fig. 3b and d), and coarsens some crystals into elongated laths. Contrast-matching small-angle neutron scattering studies also indicate a conformal coating of RuO₂ particles on the silica surface (as well as particle growth upon annealing). The RuO₂||SiO₂ aerogel composite retains the free volume and surface area of the substrate aerogel, so the approximately 1 mS cm⁻¹ bulk conductivity of the oxygen-annealed composite, which is defined by the geometric area of the SiO₂ aerogel, underestimates the conductivity of the RuO₂ nanowire.

The cryogenically deposited RuO₂ covers only about 1% of the available aerogel surface (calculated on the basis of the mass of RuO₂ deposited and assuming a 5-nm spherical particle shape). Repeating the deposition and annealing process more than doubles the mass of RuO₂ in the composite, but does not increase the electronic conductivity. These results are consistent with the formation of a nanoscopic, three-dimensional web of RuO₂ crystals with multiple paths throughout the monolith, as shown in Fig. 3c. We attribute the advantageous self-wiring that is exhibited in this process to the known autocatalytic decomposition of RuO₄ on a RuO₂ surface²¹ and to the slow kinetics of deposition at the cryogenic temperatures. Once a ruthenia nucleation centre is generated (presumably due to adsorption at a silica defect site), growth is preferential from that site. The preference of RuO₄ decomposition off a RuO₂ surface relative to a SiO₂ surface is indicated by the approximately 9:1

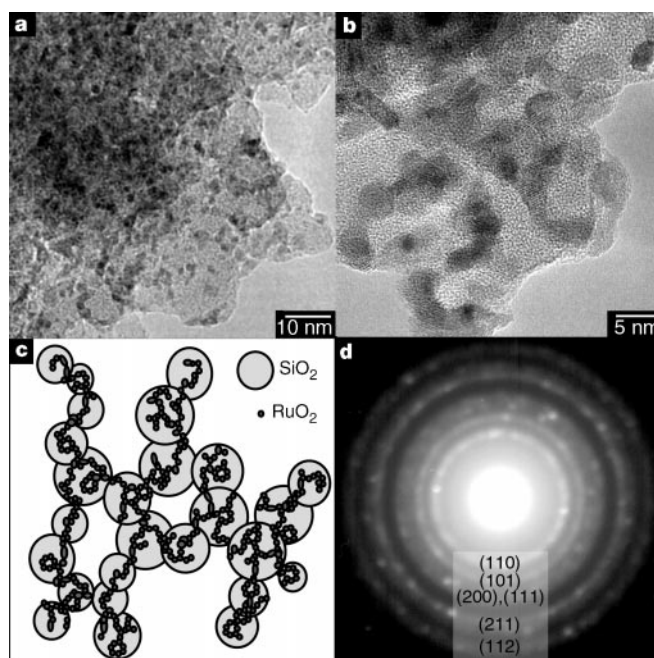


Figure 3 Transmission electron micrographs and schematic of RuO₂||SiO₂ aerogel composites. The RuO₂ particles (dark grey) form rings around the amorphous SiO₂ particles (light grey). **a**, As-deposited RuO₂ on partially densified aerogel by the cryogenic decomposition of RuO₄; **b**, Twice-coated (cryogenically deposited RuO₂/annealed under

oxygen/re-coated/re-annealed); **c**, Schematic of the self-wired path taken by the cryogenically deposited RuO₂ nanoparticles; **d**, Electron diffraction pattern, indexed to rutile RuO₂, of a once-coated sample, annealed to 145 °C.

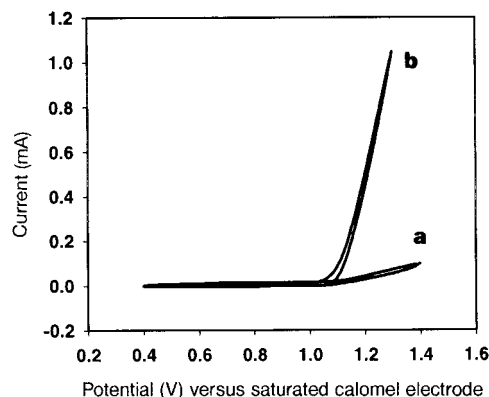


Figure 4 Electrocatalysed oxidation of chloride at a $\text{RuO}_2/\text{SiO}_2$ aerogel composite electrode. Cyclic voltammetric measurements in 1 M NaCl to measure the current that flows as the potential is linearly varied at 10 mV s^{-1} . **a**, Voltammetric response of the carbon-wax substrate; **b**, Voltammetric response of 0.55 mg of a RuO_2 -coated SiO_2 aerogel composite pressed onto the surface of the carbon-wax composite.

weight uptake for a once-coated $\text{RuO}_2/\text{SiO}_2$ composite relative to an uncoated silica aerogel of comparable surface area.

The $\text{RuO}_2/\text{SiO}_2$ aerogel composite not only demonstrates electronic properties consistent with the metallic character of anhydrous RuO_2 , but electrocatalytic activity as well. In NaCl electrolyte, $\text{RuO}_2/\text{SiO}_2$ is catalytically active for the oxidation of chloride ion to molecular chlorine gas, as seen in Fig. 4, which contrasts the voltammetric response of the composite with that of the carbon-wax substrate used to make electronic contact to the aerogel. The mesoporous network permits ready flux of the redox solute (Cl^-) into the aerogel interior and the high surface area of RuO_2 acts as an effective catalytic electrode. The current densities achieved at moderate overpotentials are about one order of magnitude less than for a conventional RuO_2 electrode²², based on the estimated RuO_2 surface area in the $\text{RuO}_2/\text{SiO}_2$ composite. However, the form of the catalytic surface on conventional dimensionally stable anodes is that of a defective, hydrous oxide (RuO_xH_y), which is about 10 times more catalytically active than anhydrous RuO_2 (ref. 22), so the area-normalized activity of $\text{RuO}_2/\text{SiO}_2$ indicates that almost all deposited RuO_2 anhydrous nanocrystallites are electrochemically accessible. Because the nanoweb of anhydrous RuO_2 deoxygenates at modest temperatures under low partial pressures of oxygen, the $\text{RuO}_2/\text{SiO}_2$ porous electrode can readily be converted to a hydrous, more catalytically active nanoweb of RuO_xH_y .

Our approach of solution infiltration of RuO_4 combined with the subzero reaction allows us to unite the high surface area and porosity of the SiO_2 aerogel host and the electronic properties of the secondary RuO_2 phase. Because anhydrous RuO_2 is a thick-film resistor, as well as a barrier layer in DRAM capacitors, we also recognize that $\text{RuO}_2/\text{SiO}_2$ aerogel composites are potential platforms to design a new type of electronic circuit—one that is nanoscopic, massively parallel over enormous surface areas, and contained within a small volume element. It may now be possible to explore inorganic equivalents to neural systems with domains, rather than elements, that process electrical information. □

Methods

Silica aerogel synthesis

Silica aerogels were prepared by base-catalysed hydrolysis and condensation of tetramethoxysilane in a procedure reported in detail²³. Dried aerogels were partially densified²⁴ by heating to 900°C at 2°C min^{-1} to strengthen the substrate to immersion in liquids. The partially densified aerogel is about 80% porous and, based on N_2 -physisorption measurements, retains a relatively high surface area ($400\text{--}500 \text{ m}^2 \text{ g}^{-1}$), with the primary loss of

surface area due to collapse of the micropores²⁵. Aerogel substrates were 1–3-mm thick disks as shaped by grinding with dry 600-grit silicon carbide paper.

RuO_2 deposition

Solutions of RuO_4 in pentane, rather than water, were used to minimize capillary forces on the aerogel network during re-wetting and re-drying at subcritical conditions. Up to four partially densified aerogel substrates weighing a total of about 100 mg were placed in a round-bottomed flask with a sidearm, evacuated to 5×10^{-3} mtorr, and back-filled with N_2 . Approximately 2–3 ml of purified pentane was condensed in the sidearm, then warmed to room temperature and allowed to equilibrate with the aerogel. By slightly cooling the flask, the pentane condensed in the flask and covered and filled the aerogel pieces. 10 ml of pentane was used to extract RuO_4 from aqueous RuO_4 (10 ml of 0.5 wt. % solution, Strem Chemicals) and added to the flask containing the aerogel pre-cooled to -78°C in a dry ice/acetone bath. All but 2–3 ml of pentane was removed by vacuum distillation and the bath and sample were allowed to warm gradually to ambient temperature over a period of 2–3 days. Based on periodic visual inspection, the sample changed from transparent to black at about -35°C , corresponding to the initial conversion of RuO_4 to RuO_2 . After the sample reached room temperature, the flask was cooled to -78°C and the remaining pentane was removed by vacuum distillation.

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Determining the temperature of petroleum formation from the kinetic properties of petroleum asphaltenes

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Knowledge of the timing and location of petroleum formation is important in assessing the extent of available reserves in hydrocarbon-forming basins. This can be predicted from the thermal history of a basin and the kinetic parameters that characterize the thermal breakdown of kerogen in source rocks. At present, the kinetic parameters of kerogen breakdown are experimentally determined using immature rock samples from basin margins¹, but questions remain about the accuracy of this approach², especially when significant variability is observed within individual source units^{3–5}. Here we show that the kinetics of hydrocarbon generation from petroleum asphaltenes can be used to determine the temperature conditions of the actual source rock at the time of expulsion of the sampled petroleum. This relationship reflects the structural similarity of asphaltenes to the parent kerogen^{6,7}. We expect that our approach may be used as a comparatively simple alternative method for assessing the petroleum generation characteristics of a given basin, which will allow for better estimates of the available oil resources and the risks associated with their exploration.

Asphaltenes are organic macromolecular aggregates that make up 0–40% of non-biodegraded petroleum and up to more than 80% of source rock bitumen⁸; they are most abundant in low-maturity or biodegraded black oils. Although their detailed structure is poorly understood, it is widely held that asphaltenes in source rocks bear close structural similarities to their precursors, kerogens^{6,7}, and that this structural signature is carried within asphaltenes expelled from source rocks during petroleum migration⁹.

Asphaltene geochemistry has been widely used in petroleum exploration and production. Examples include analysis of biomarker skeletons in asphaltenes for correlating crude oils and inferring likely sources^{10,11}, asphaltene chemical and physical properties for defining permeability barriers in reservoirs¹², and asphaltene pyrolysis products for the prediction of petroleum gas–oil ratios^{13–15}.

Petroleum asphaltenes have not, until now, been used in the kinetic modelling of bulk hydrocarbon generation. Instead, studies have focused on source rocks, with kinetic parameters usually being determined on available immature samples. The success of the prediction depends strongly on how closely the samples resemble the actual source rocks which generated the petroleum, which is difficult to assess, owing to vertical and lateral source facies variability^{3–5}. It would be extremely useful if kinetics of petroleum generation were determined using asphaltenes from the oil itself since the parameters can be related to actually active source rocks rather than just putative source rocks.

We have already shown that it is technically feasible to measure kinetic parameters using asphaltenes¹⁶. Here we report on their utility in petroleum exploration. First, the concepts involved are illustrated using a suite of marine carbonate (sulphur-rich) source rocks and crude oils from the Sonda de Campeche (SDC), in the southern Gulf of Mexico, where maturity is the main control of petroleum composition and source facies variations are minor^{17,18}. As discussed elsewhere^{17–19}, petroleum accumulations in Palaeocene reservoirs originate in distinct generation pulses from the Tithonian

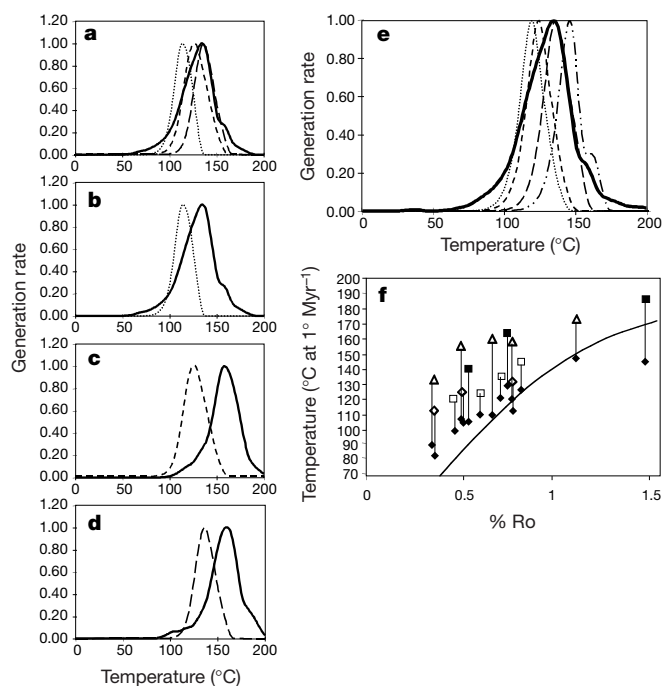


Figure 1 Comparison of computed transformation ratios of source-rock kerogens and asphaltenes. Asphaltenes were from source rocks of increasing maturity (**a–d**) and petroleum asphaltenes (**e**) were from oils of increasing maturity from the Sonda de Campeche using a heating rate of 1 °C Myr^{−1}. **a**, Comparison of the asphaltene kinetic predictions for samples of increasing maturity with the lowest maturity kerogen sample. Solid line, source-rock kerogen; dotted line, 0.35% Ro; short-dashed line, 0.49% Ro; long-dashed line, 0.75% Ro. **b–d**, Comparison of the asphaltene kinetic predictions with those of the parent kerogen for samples of increasing maturity. **b**, 0.35% Ro; **c**, 0.49% Ro; **d**, 0.75% Ro. (lines as in **a**). **e**, **f**, Comparison of kinetic predictions from source rock and petroleum asphaltenes. **e**, Comparison of computed transformation ratios (TR) of source-rock kerogen and asphaltenes from petroleum of increasing maturity. Solid line, source-rock kerogen; dotted line, 0.45% Ro; short-dashed line, 0.59% Ro; long-dashed line, 0.70% Ro; long-dash/two-short-dashed line, 0.80% Ro. **f**, Calculated T_{max} of Campeche kerogen (triangles), source rock asphaltenes (diamonds) and petroleum asphaltenes (squares) and their respective 5% TR temperatures (diamond at the base of each line) as a function of sample maturity. The solid line is the calculated vitrinite reflectance at a heating rate of 1 °C Myr^{−1}. Kerogen data from a Toarcian source rock maturity sequence (Hilsmulde, Germany²⁰) is also included for comparison (black squares).

Table 1 Samples from the Sonda de Campeche, Mexico

Sample	Source-rock kerogen	Source-rock asphaltene	Petroleum asphaltene	Maturity
E 41495	X	X		0.35% Ro
E 41449	X	X		0.49% Ro
E 41488	X	X		0.75% Ro
E 41459	X			0.65% Ro
E 41500	X			1.09% Ro
E 41531			X	0.45% Rc
E 41532			X	0.59% Rc
E 41543			X	0.70% Rc
E 41541			X	0.80% Rc

The Tithonian source rocks of Mexico were deposited in a restricted marine environment, and are rich in organic carbon. Although minor facies variations could be determined by microscopy, homogeneity in a chemical sense is indicated by Rock-Eval, pyrolysis-gas chromatography and biomarker data^{17,18}. Maturity increases from northeast to southwest across the area. The full maturity range extends from Ro = 0.4–1.3%, but only a part of this is used in the reported sample set. Ro, measured vitrinite reflectance; Rc, calculated vitrinite reflectance¹⁷.

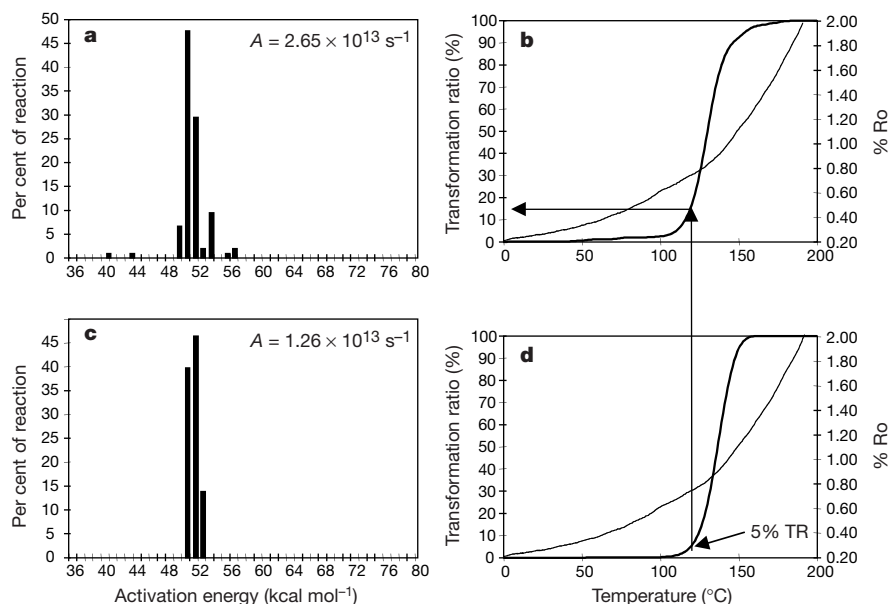


Figure 2 Activation energy distributions and calculated transformation ratios. **a, b**, Source-rock kerogen correlated to the 2/2-5 oil. **c, d**, Petroleum asphaltenes from the 2/2-5 oil. Arrows on the TR diagrams indicate inferred temperature of oil-phase generation and

expulsion (120 °C) as determined from the 2/2-5 petroleum asphaltene kinetics (**d**) and extent of transformation of the kerogen based on this temperature (15% TR, **b**). Thick line, TR; thin line, computed % vitrinite reflectance (Ro).

source rock over narrow maturity ranges, rather than a continuous cumulative sum. The samples used and their maturity are listed in Table 1. Examples from the Central and Viking Grabens of the Norwegian continental shelf are then presented.

The asphaltene precipitation method used has been described previously¹⁶. Kinetic analyses were performed using the Source Rock Analyser at five different heating rates (1, 5, 15, 30 and 50 °C min⁻¹). Measured generation rate versus temperature curves were analysed, assuming a distribution of activation energies and a single frequency factor. Asphaltene constitute a compound solubility class^{20,21} and, hence, include a wide variety of compounds of differing molecular weight. Some of these are thermovaporized at low temperatures in the heating procedure, whereas others are involatile and break down in cracking reactions at higher temperatures. Only pyrolysis reactions near the maximum generation rate temperature T_{max} of the sample were used, as they are directly comparable with the thermal degradation of kerogen in whole-rock pyrolysis.

As the maturity of Tithonian source rocks (SDC) increases, labile substituents are progressively cleaved from the kerogen structure, this being indicated by increasing values of average activation energy and frequency factor. This confirms earlier results on other marine source rocks²². When applied to a heating rate of 1 K Myr⁻¹, the kinetic parameters predict a shift in generation curves to higher temperatures, as shown in Fig. 1a–d. Asphaltene from the same sample series also show a shift in generation curves to progressively higher temperatures with increasing maturity level (Fig. 1a–d). We deduce that cracking reactions, selectively involving the more labile components of the macromolecular structure, result in progressive changes in the compositions of both genetically related asphaltene and kerogen. For each of the three asphaltene–kerogen pairs, the rate curve for the asphaltene averages a lower T_{max} than that of the respective kerogen. Hence, the asphaltene structures cannot be considered to be representative of the whole kerogen. Instead, the asphaltene appears to be equivalent to the most labile part of the respective kerogen. This has a bearing on how petroleum asphaltene kinetics can be used to predict the timing of petroleum generation. But first, we show that the structural information in asphaltene is transferred without fractionation from source rocks

to migrating petroleum.

Figure 1e shows generation curves calculated from four petroleum asphaltene of increasing maturities in the SDC (Table 1). Asphaltene in petroleum that are generated and expelled at low levels of maturity possess more labile constituents than do those that are generated and migrate later. This reflects the trend shown by the source rocks. Moreover, the asphaltene from both source rocks and crude oils exhibit the same relationship between vitrinite reflectance Ro (measured for rocks and calculated for crude oils¹⁷) and the predicted T_{max} as illustrated in Fig. 1f. These results prove that the kinetic signature of the source-rock asphaltene at any of the given maturity levels is retained by expelled petroleum and that secondary migration has no effect on kinetic parameters.

We now use asphaltene kinetics for predicting the occurrence of petroleum in time and space. We consider the yield curve predicted from the 0.59% Ro petroleum asphaltene in Fig. 1e. We know that this evolution pattern is transferred into migrating petroleum (Fig. 1f), and that the curve in Fig. 1e represents the breakdown of the asphaltene into volatile products. It therefore does not reflect the generation curve of the petroleum in which the asphaltene was found; instead, it is a prediction of later stages of thermal alteration not yet attained by its source rock. We can, however, use the onset of this curve as an indication of the maximum temperature that prevailed when the asphaltene was generated from its precursor kerogen. The 5% transformation rate (TR) value determined from the asphaltene generation-rate curve is the one we use for the characterization of the expulsion temperature. Figure 1f shows, in addition, T_{max} and 5% TR values determined from the bulk kerogen kinetics of SDC kerogens of increasing maturity (Table 1) and from a siliciclastic source-rock maturity sequence from Germany²². The kerogen T_{max} data shows a continuous evolution towards higher values as a function of maturity, independent of source-rock type. The 5% TR values of the kerogens, however, match those determined for the asphaltene very closely. The 5% TR values also correlate closely to the calculated vitrinite reflectance for the heating rate used (1 °C Myr⁻¹). This implies that asphaltene kinetic data can be used directly for estimation of the generation/expulsion temperature of

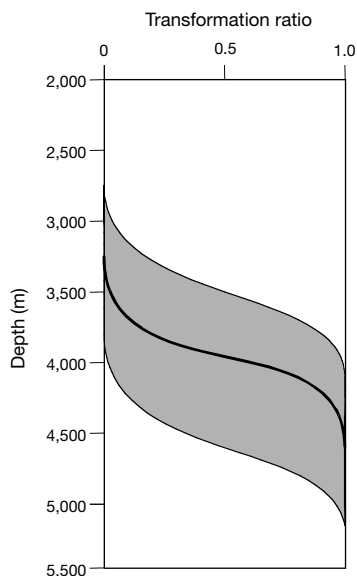


Figure 3 Range of kinetic variability of Draupne Formation samples compared to petroleum asphaltene. Data is plotted as a function of source rock burial depth. Draupne Formation samples, shaded area; kinetic predictions, black line. Generation and expulsion of the sampled petroleum phase can be inferred to have occurred at a depth of 3,600 m (5% TR). Modelled results using a calibrated burial history of a pseudo well in the active kitchen area of Snorre Field. Asphaltene kinetics were determined on eight different oil samples from Snorre Field. Kinetic variability between the individual asphaltene samples was almost non-existent even on a field scale (variation range in 0.5% TR temperature predictions of the individual asphaltenes was under 1 °C).

the petroleum phase sampled, at least for oils derived from a single source rock.

Two examples from the Norwegian continental shelf will illustrate the utility of the method. Well 2/2-5 (Central Graben, Norwegian North Sea) tested an oil of singular geochemical characteristics (high isoprenoid and gammacerane contents and a very heavy isotopic signature²³), which differed so greatly from surrounding discoveries that the original long-range migration scenario had to be discarded. The Upper Jurassic source-rock sequences were immature, according to traditional understanding, so an alternative, possibly deeper, source was suggested. However, oil source-rock correlation linked the 2/2-5 oil to a specific rich and oil-prone interval of the upper Jurassic Farsund formation²³. An immature sample was subjected to kinetic analysis and revealed a narrow and relatively low activation energy distribution. Calculation of the generation curves under geological conditions demonstrated that this kerogen type could enter the oil window at depths up to 500 m shallower than the average Upper Jurassic Mandal formation. Hence, the local kitchen (source rock pod) area of 2/25 is in fact generating oil. The source-rock sample used for this kinetic analysis had been sampled at a well location quite far from the 2/2-5 area²³, so that the question was raised as to the validity of the extrapolation. Analysis of the 2/2-5 oil asphaltenes revealed a nearly identical activation energy distribution and frequency factor, as shown in Fig. 2. Determination of the 5% TR threshold of the asphaltene sample in Fig. 2 allowed the characterization of the expulsion temperature.

Snorre Field, located in blocks 34/4 and 34/7 of the Norwegian North Sea, provides the second example. The Upper Jurassic Draupne Formation is the main, if not only, source rock contributing hydrocarbons to Snorre Field²⁴. The Draupne Formation is, however, characterized by an extreme variability in kinetic parameters⁵ ($T_{\max}$ variation of up to 40 °C at geologic heating rates, M. Erdmann personal communication). Accordingly, the

mapping of active kitchen areas for Snorre Field has been extremely difficult. Figure 3 compares the range of kinetic variability determined on the Draupne Formation samples with the asphaltene kinetics of the Snorre petroleum as a function of depth using the burial history of the kitchen areas. The 5% TR value from the asphaltene kinetic data (reached at 3,600 m depth) allows us to accurately map the extent of the kitchen area which has met the time/temperature conditions required for the generation of the oil phase encountered in Snorre Field.

Thus, the kinetics determined from petroleum asphaltenes reflect the extent of thermal stress which the source rock has been subjected to when the petroleum was generated. These kinetics can be used in conjunction with a reconstructed burial and thermal history of an area to determine where and when the petroleum phase was generated and expelled. We consider this approach, of determining the generation/expulsion and time/temperature conditions using kinetics based on actual data from the oil encountered, to be a significantly better method for reducing the risk in maturity assessment than the use of kinetic data extrapolations. □

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Anomalous ^{17}O compositions in massive sulphate deposits on the Earth

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The variation of $\delta^{18}\text{O}$ that results from nearly all physical, biological and chemical processes on the Earth is approximately twice as large as the variation of $\delta^{17}\text{O}$. This so-called 'mass-dependent' fractionation is well documented in terrestrial minerals^{1,2}. Evidence for 'mass-independent' fractionation ($\Delta^{17}\text{O} = \delta^{17}\text{O} - 0.52\delta^{18}\text{O}$), where deviation from this tight relationship occurs, has so far been found only in meteoritic material and a few terrestrial atmospheric substances³. In the rock record it is thought that oxygen isotopes have followed a mass-dependent relationship for at least the past 3.7 billion years (ref. 4), and no exception to this has been encountered for terrestrial solids⁵. Here, however, we report oxygen-isotope values of two massive sulphate mineral deposits, which formed in surface environments on the Earth but show large isotopic anomalies ($\Delta^{17}\text{O}$ up to 4.6‰). These massive sulphate deposits are gypcretes from the central Namib Desert and the sulphate-bearing Miocene volcanic ash-beds in North America. The source of this isotope anomaly might be related to sulphur oxidation reactions in the atmosphere and therefore enable tracing of such oxidation. These findings also support the possibility of a chemical origin of variable isotope anomalies on other planets, such as Mars⁶.

We have analysed sulphates from laboratory experiments and natural sources (Fig. 1). The $\delta^{18}\text{O}$ and $\delta^{17}\text{O}$ of seawater sulphate, evaporites, sulphate from microbial sulphate reduction experiments, and sulphates formed from mineral-sulphide oxidation in air or soils, all fall on the mass-dependent terrestrial fractionation line, given by the relationship $\delta^{17}\text{O} = 0.52\delta^{18}\text{O}$. The deviation from this relationship, defined as $\Delta^{17}\text{O} = \delta^{17}\text{O} - 0.52\delta^{18}\text{O}$, is approximately zero ($-0.04 \pm 0.05\text{‰}$, $n = 36$). The central Namib Desert gypcretes and the Miocene volcanic ash-falls in the western United States, however, possess sulphate $\delta^{18}\text{O}$ and $\delta^{17}\text{O}$ values that deviate from the terrestrial fractionation line. The sulphate $\Delta^{17}\text{O}$ of Namib gypcretes ranges from 0.20‰ to 0.51‰ (Table 1), well outside the experimental error of $\pm 0.05\text{‰}$. Gypsum and other water-soluble sulphate minerals from Miocene volcanic ash deposits in Nebraska and South Dakota have strikingly large $\Delta^{17}\text{O}$ values, up to 4.59‰, in contrast to the zero or slightly positive $\Delta^{17}\text{O}$ found in adjacent

Table 1 Isotopic compositions and occurrences of the Namib gypcretes

Sample	$\delta^{18}\text{O}$	$\delta^{17}\text{O}$	$\Delta^{17}\text{O}$	Depth below surface (cm)
GOR13-2	12.4	6.8	0.34	5
GOR13-3	8.3	4.6	0.33	16
GOR13-5	10.5	5.9	0.42	36
GOR13-6	11.6	6.4	0.35	52
GOR14-6	12.2	6.6	0.29	40
GBB16-6	11.8	6.4	0.26	45
GBB17-5	9.8	5.3	0.20	16
GBB17-6	9.5	5.2	0.26	46
GBB17-8	11.3	6.1	0.26	66
AUS6-5	9.9	5.6	0.40	10
SWA6-1	12.6	7.0	0.40	1
SWA6-2	12.0	6.6	0.31	9 (crack)
SWA6-3	11.4	6.3	0.34	5
SWA6-4	13.2	7.3	0.40	24
SWA6-6	13.0	7.1	0.37	50
SWA6GYP	11.1	6.2	0.38	52
SWA6-7	10.1	5.8	0.51	70
SWA9-10	11.3	6.1	0.23	46

Samples from the same soil profile are grouped together. Soil profiles are listed with decreasing distance from the ocean. Isotopic compositions are given in SMOW. $\delta^{18}\text{O} = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1,000\text{‰}$, where R is the number ratio $^{18}\text{O}/^{16}\text{O}$.

soil and fluvial horizons (Table 2).

Sulphate minerals with $\Delta^{17}\text{O} \approx 0\text{‰}$ are expected because thermodynamic and kinetic (including biological) processes such as evaporation, mineral-sulphide oxidation (by Fe^{3+} or air O_2), and sulphate reduction generate mass-dependent compositions (Fig. 1). Therefore, sulphate minerals with positive $\Delta^{17}\text{O}$ values, such as those found in the central Namib Desert gypcretes and the Miocene volcanic ash-falls in the western United States require a different process. The only documented terrestrial reservoirs with positive $\Delta^{17}\text{O}$ are from the atmosphere. Oxidants such as O_3 and H_2O_2 are known to have positive $\Delta^{17}\text{O}$ values that range from 1 to more than 25‰ (refs 7–10). Others, like OH and NO_x , remain to be measured.

Tropospheric O_3 and H_2O_2 in rainwater may transfer their positive $\Delta^{17}\text{O}$ values to the product sulphate by *in situ* oxidation of surface minerals (for example, sulphides). Our measurement of the sulphate produced by marcasite (FeS_2) oxidation in the air yielded $\Delta^{17}\text{O} \approx 0\text{‰}$, indicating that the *in situ* pathway is not the major source of positive $\Delta^{17}\text{O}$. A more likely source is the wet and dry atmospheric deposition of sulphate produced by atmospheric oxidation of reduced gaseous sulphur compounds. The anomaly can come from atmospheric oxidants such as O_3 , H_2O_2 or OH radicals as a result of aqueous or gas-phase S(IV) oxidation. A similar explanation was invoked to interpret positive $\Delta^{17}\text{O}$ values (0.20‰ to 1.80‰) observed in aerosol and rainwater sulphate^{11,12}. Although the mechanisms of these atmospheric processes are still subjects of intensive study, the connection between our $\Delta^{17}\text{O}$ -positive sulphate minerals and atmospheric oxidation processes is unequivocal, as shown by the close association with a high flux of atmospheric reduced sulphur compounds in our two reported cases.

Gypcrete soils in the central Namib Desert occur extensively near the coast and gradually thin off and disappear at about 50–70 km from the coast, constituting one of the most extensive gypsum accumulations in Africa. On the basis of $\delta^{34}\text{S}$, meteorological, hydrological, and geological information, Eckardt and Spiro¹³ suggest that sulphate in the Namib Desert originates mostly from biologically produced marine sulphur (that is, not derived from sea salt), particularly the oxidation of marine dimethyl sulphide (DMS). This conclusion is also supported by the lack of correlation between gypcrete accumulation and bedrock in this location and the positive correlation between gypcrete accumulation and the proximity to the ocean, a source of DMS. The Benguela Current,

Table 2 Description of the Miocene volcanic ash-bed deposits

Sample	$\delta^{18}\text{O}$	$\delta^{17}\text{O}$	$\Delta^{17}\text{O}$	$\delta^{34}\text{S}$	Description
Loope-1	12.5	11.1	4.59	0.67	Sand crystal from Scotts Bluff, Nebraska
Loope-2	12.1	10.6	4.36	0.98	As above; different crystal
BNP-27	10.9	8.5	2.84	1.72	Silt/sandstone in the Sharp Formation, North Trail, BNP
BNP-SP-1	9.6	7.7	2.72	–	Rockyford Ash bed, Saddle Pass, BNP
BNP-SP-2	10.6	8.5	2.98	4.29	As above; different specimen
BNP-25-1	12.6	6.8	0.25	7.45	Channel deposits in the Sharps Formation, North Trail, BNP
BNP-28	–0.9	–0.5	–0.02	–6.93	Clay/siltstone associated with vertebrate fossils in the Sharps Formation, North Trail, BNP

Samples from Badlands National Park (BNP), South Dakota, are listed in stratigraphically descending order. Sulphate-oxygen and sulphur-isotopic compositions are given in SMOW and CDT, respectively.

which originated in the early Late Miocene (about 10 million years ago) off the west coast of South Africa and Namibia, provides upwelled waters extremely rich in phosphate, nitrate and silica, which support a rich population of phytoplankton¹⁴ and a high DMS concentration in the local atmosphere. According to this interpretation, the anomalous $\Delta^{17}\text{O}$ of the Namib gypcretes reflects chemistry associated with atmospheric oxidation of DMS.

Volcanic activity reached its maximum during the Oligocene in western North America¹⁵. Layers of volcanic-associated deposits are exposed throughout the Oligocene/Miocene continental deposits in

eastern Wyoming, South Dakota, North Dakota and Nebraska^{16,17}. The current water-soluble sulphate content is about 0.02% in the Miocene Rockyford ash-bed (sample BNP-SP). It is well known that volcanic ash contains a significant amount of sulphate, occurring as sulphuric acid droplets that react to form gypsum or other sulphate minerals¹⁸. The occurrence of gypsum pseudomorph in the sandy Gering Formation may suggest high sulphate content in overlaid ash-beds. A volcanic origin for the sulphate sulphur is supported by its juvenile $\delta^{34}\text{S}$ values (Table 2). The large sulphate $\Delta^{17}\text{O}$ signatures from these widespread Miocene ash-beds indicate a link between the $\delta^{17}\text{O}$ -anomaly and volcanic processes. Some of the sulphate $\Delta^{17}\text{O}$ values are more positive than has been observed in sulphate aerosol, rainwater sulphate, and desert gypcrete. We expect the sulphate $\Delta^{17}\text{O}$ value in volcanic ash will vary depending on distance from source and the nature of eruption.

Sulphate is one of a few non-labile oxygen-bearing ions (aquametal ions or oxo-anions)¹⁹ that, once produced, are resistant to oxygen exchange with other ambient oxygen-bearing compounds (for example, water) under most surface environments. This chemical feature is essential for the preservation of the anomalous $\Delta^{17}\text{O}$ signature in sulphate minerals.

The discovery of positive $\Delta^{17}\text{O}$ values in minerals formed on Earth has important implications in Earth science. First, this is the first, to our knowledge, demonstration of a transfer of $\delta^{17}\text{O}$ anomaly from atmosphere to crustal minerals. At this time, only a few approaches are available for probing ancient atmospheric conditions^{20–22}. Sulphate $\Delta^{17}\text{O}$ has the potential to be a tracer for ancient atmospheric ozone activity, chemistry in volcanic plumes, and the sulphur biogeochemical cycle in desert environments, where atmospheric sulphate is a major sulphur component. Second, it is interesting to evaluate recent findings of anomalous $\Delta^{17}\text{O}$ for water, sulphate, and carbonate in SNC meteorites relative to coexisting igneous silicate minerals^{6,23,24}. We document here a larger-magnitude $\Delta^{17}\text{O}$ signature in terrestrial sulphate minerals than have been observed in SNC meteorites. Our observations are consistent with an atmospheric chemical origin for the SNC oxygen-isotope systematics. To establish the full implications of the $\delta^{17}\text{O}$ anomalies found in terrestrial minerals, however, requires a fundamental understanding of the sulphur oxidation processes occurring in the atmosphere. The measurements of isotopic fractionation factors for the relevant oxidation reactions will permit quantification of the observations, as has been done for many other species³. □

Methods

Barite is precipitated from filtered and acidified solutions, which contain water-soluble sulphate from crushed gypcretes, sand crystals, volcanic ash-beds, and fluvial deposits. Molecular oxygen is extracted directly from barite following a newly developed method²⁵, which employs a 30 W CO_2 -laser fluorination technique. Fine-grained barite was heated in a BrF_3 atmosphere, and more than 90% of the samples were analysed in duplicate. Samples are often loaded and analysed together with other $\delta^{17}\text{O}$ -normal barites in one set. O_2 was run on a Finnigan MAT 251. We found no evidence for mass interference by NF_3 fragments or S compounds in our analyses. The samples with large positive $\Delta^{17}\text{O}$ values were purified and reanalysed using a method²⁶ that quantitatively removes these compounds, after initial mass spectrometric analysis. No differences in $\Delta^{17}\text{O}$ were observed. The results are highly reproducible and the difference between duplicate analyses is better than $\pm 0.7\text{‰}$ and $\pm 0.05\text{‰}$ for $\delta^{18}\text{O}$ and $\Delta^{17}\text{O}$, respectively.

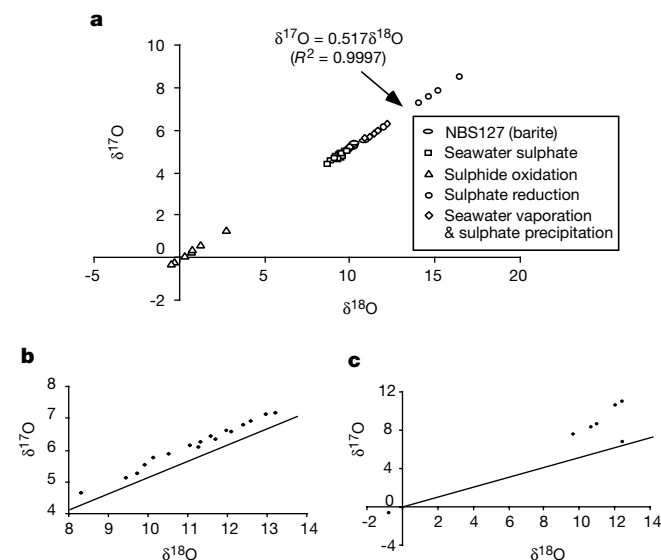


Figure 1 $\delta^{17}\text{O}$ versus $\delta^{18}\text{O}$ for various sulphates. **a**, A fractionation line defined by major sulphate-involving processes on Earth. The seawater sulphate consists of sea water from La Jolla, California, and seawater sulphate adsorbed on the surface of amorphous and poorly crystalline ferric oxides from Red Seamount, Pacific Ocean. Sulphate from the oxidation of mineral-sulphides includes several non-evaporative gypsum minerals from glacial-till deposits in Akron, Ohio, and water-soluble sulphate from surface oxidation of marcasite (FeS_2) exposed to air. The sulphate reduction experiment was conducted by adding organic-rich soil into a sealed tank of La Jolla sea water and allowing time for fractionation of oxygen isotopes by microbial sulphate reduction. Gypsum, other sulphate-bearing evaporitic minerals, and sulphate from final residual solution were collected from two seawater evaporation experiments conducted at 22 °C and 55 °C, respectively. **b**, Gypcrete and gypsum-bearing soils were collected from different soil horizons and different localities in the central Namib Desert. **c**, Volcanic ash and associated deposits were from the Miocene Arikaree Group, Nebraska and South Dakota (see Table 1 and Table 2 for details). The straight line in **b** and **c** is the terrestrial fractionation line ($\delta^{17}\text{O} = 0.528\delta^{18}\text{O}$). The error bar (about ± 0.7 for $\delta^{18}\text{O}$) was not plotted on the diagrams. Due to the high correlation between $\delta^{17}\text{O}$ and $\delta^{18}\text{O}$ during mass spectrometry analysis, the error for $\Delta^{17}\text{O}$ is very small (less than ± 0.05). Thus, a thin rod with slope of 0.52 would best represent the actual point on the diagram. Delta values are given in SMOW.

Most samples give 28–33% O₂ yield during laser fluorination. We found by analysing NBS127 (NIST sulphate standard), seawater sulphate, and a low- $\delta^{18}\text{O}$ in-house standard that the raw $\delta^{18}\text{O}$ values are consistently 9.4 lower than the reported ones, owing to the incomplete O₂ generation. The correction factor is also verified by more than a dozen natural sulphate samples—that have been analysed using both the laser-fluorination method and the graphite reduction/CO₂-fluorination method²⁷—that normally reach a 85% to 100% O₂ yield in our laboratory. This comparison also verifies that the incomplete O₂-generation using a CO₂-laser does not deviate from the slope of 0.52. Thus, the reported $\delta^{17}\text{O}$ value is increased by 4.89‰. $\delta^{34}\text{S}$ was analysed using the SF₆ method²⁸. In this report, all $\Delta^{17}\text{O}$ values are calculated on the basis of raw $\delta^{17}\text{O}$ and $\delta^{18}\text{O}$ values. We use the linear equation to calculate $\Delta^{17}\text{O}$ because all the raw $\delta^{17}\text{O}$ and $\delta^{18}\text{O}$ values are close to the vicinity of the origin and the range of $\delta^{17}\text{O}$ and $\delta^{18}\text{O}$ values among different samples are small.

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Determining multiple length scales in rocks

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Carbonate reservoirs in the Middle East are believed to contain about half of the world's oil¹. The processes of sedimentation and diagenesis produce in carbonate rocks microporous grains and a wide range of pore sizes, resulting in a complex spatial distribution of pores and pore connectivity². This heterogeneity makes it difficult to determine by conventional techniques the characteristic pore-length scales, which control fluid transport properties. Here we present a bulk-measurement technique that is non-destructive and capable of extracting multiple length scales from carbonate rocks. The technique uses nuclear magnetic resonance to exploit the spatially varying magnetic field inside the pore space itself—a 'fingerprint' of the pore structure. We found three primary length scales (1–100 μm) in the Middle-East carbonate rocks and determined that the pores are well connected and spatially mixed. Such information is critical for reliably estimating the amount of capillary-bound water in the rock, which is important for efficient oil production. This method might also be used to complement other techniques^{3–5} for the study of shaly sand reservoirs and compartmentalization in cells and tissues.

Nuclear magnetic resonance (NMR) is often used to investigate porous media by applying external magnetic field gradients in a fashion analogous to X-ray diffraction in determining the size of cells⁶ and pores^{7–9}. In fact, when a sample is placed in a uniform magnetic field, a spatially varying field and its gradients appear naturally inside the pore space as a result of the magnetic susceptibility contrast between the host solid material and the pore-filling fluid¹⁰. This field is called the internal field, B^i , and it can be large enough in natural materials such as rocks^{11–14} to interfere with the

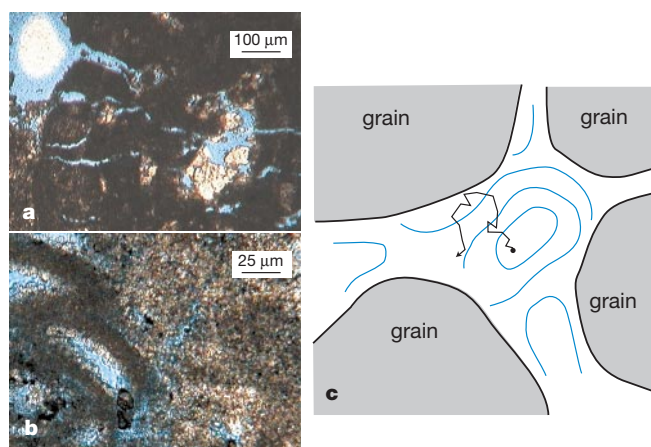


Figure 1 Thin-section micrographs of the pore space in Thamama carbonate rocks and an illustration of the internal magnetic field. **a, b**, The blue regions are pores filled with blue epoxy before sectioning. Pores of size a few μm to about 100 μm are clearly visible. The smaller pores show up as different level of shades owing to limited resolution. It is crucial to determine these length scales in understanding the transport properties. **c**, Diagram of the typical pore space, internal magnetic field B^i and diffusion. The blue lines illustrate the constant B^i contours (the component along the external field) reflecting the local pore geometry. The magnetization decay due to diffusion in B^i is used to characterize the pore sizes.

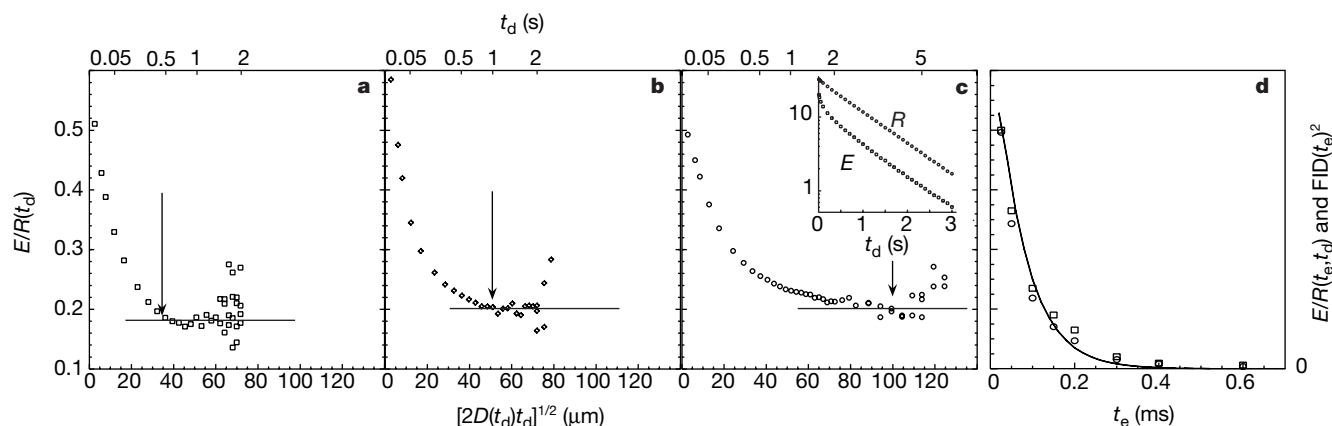


Figure 2 Experimental results on uniform-size, randomly-packed glass bead samples saturated with water. **a–c**, The DDIF ratio (E/R) as a function of the r.m.s. displacement, that is, $[2D(t_d)t_d]^{1/2}$, of water molecules calculated for each bead size using the formula in ref. 19. The corresponding t_d is shown at the top of the graphs. The average diameters are: **a**, 30.6; **b**, 50; and **c**, 105 μm . The initial decay and eventual constant DDIF ratio is

clear for all samples. The inset in **c** shows the echo (E) and the reference signals (R) for the 100- μm bead sample. **d**, The DDIF ratio as a function of t_e for the 30- μm bead sample, at $t_d = 0.4$ s (squares) and 1 s (circles) which correspond to r.m.s. displacements of 32 and 50 μm respectively. The line is the square of the FID, consistent with equation (3). NMR experiments were performed on a Tecmag spectrometer at a magnetic field of 2.14 T.

applied gradients. B^i is determined by the spatial distribution of pores and thus reflects the detailed pore structure, shown in Fig. 1. We have developed a new NMR technique to characterize B^i in order to study the complex pore geometry in carbonate rocks. We will first describe the essential concept of this method, demonstrate its utility using samples of known sizes, and then apply it to natural rocks.

We use water protons to probe B^i as they diffuse in the pore space, by measuring the ^1H magnetization diffusion with the stimulated echo pulse sequence¹⁵: $\frac{\pi}{2} - t_e - \frac{\pi}{2} - t_d - \frac{\pi}{2} - t_e - \text{echo}$. Here, t_d is the diffusion times and t_e is the echo time. The notation $\pi/2$ denotes a radio-frequency pulse that rotates the spin vector by 90° . For $t_e \ll t_d$, diffusion during t_e may be neglected. The normalized echo intensity, E , measure the change in B^i as protons diffuse during t_d (ref. 10):

$$E(t_e, t_d) = \int d\mathbf{x}_1 d\mathbf{x}_2 e^{i\gamma t_e [B_z^i(\mathbf{x}_1) - B_z^i(\mathbf{x}_2)]} P(\mathbf{x}_1, \mathbf{x}_2, t_d) \quad (1)$$

where γ is the ^1H gyromagnetic ratio. B_z^i is the component of B^i along the external field, $\mathbf{x}_1$ and $\mathbf{x}_2$ are the initial and the final positions and $P(\mathbf{x}_1, \mathbf{x}_2, t_d)$ is the diffusion propagator for the magnetization density¹⁰. No external field gradient is applied.

The additional decay of E due to spin-lattice relaxation (T_1) can be calibrated by the reference signal, $R(t_e, t_d)$, obtained in: $\frac{\pi}{2} - t_e - \pi - t_e - \frac{\pi}{2} - t_d - \frac{\pi}{2} - \text{FID}$. R is acquired as the initial amplitude of the free-induction decay (FID) signal. The π pulse cancels the phase accumulation due to B^i and thus R is independent of B^i . For the samples considered here, we focus on the decay due to diffusion in the internal field (DDIF) by using the ratio E/R (DDIF ratio) to remove the effect of the spin-lattice relaxation.

Experimental results using random-packed bead samples (Duke Scientific Co.) are shown in Fig. 2. For all samples, the DDIF ratio shows a progressive decay as a function of t_d , at short t_d . This decay is caused by the change of B^i for each diffusing spin, $[B_z^i(\mathbf{x}_1) - B_z^i(\mathbf{x}_2)]^2 \neq 0$, similar to the case of applied field gradients¹⁵. However, the decay rate reduces as t_d lengthens, and eventually, for all bead sizes, the DDIF ratio becomes constant at long t_d . This time-dependent behaviour can be used to define a critical time t_c as the time when the decay rate approaches zero. The corresponding diffusion length is indicated by the arrows in Fig. 2a–c and it corresponds well to the bead diameter of the individual sample.

To understand the long-time asymptote of the t_d -independent signal, equation (1) may be rewritten in terms of the magnetic field

B , neglecting spin relaxation:

$$E(t_e, t_d) = \int_{B_{\min}}^{B_{\max}} dB_1 dB_2 f(B_1) f(B_2) \times e^{i\gamma t_e [B_1 - B_2]} \tilde{P}(B_1, B_2, t_d) \quad (2)$$

where $f(B)$ is the distribution of B^i and $\tilde{P}(B_1, B_2, t)$ is the propagator in B^i space. However, unlike for the applied gradients, it is crucial to realize that B^i is bounded in magnitude and the field variation occurs over the pore-length scale. When the molecule has explored the entire B^i space (at large t_d), B_1 and B_2 become uncorrelated and $\tilde{P}(B_1, B_2, t) \rightarrow 1$:

$$E(t_e, t_d \rightarrow \infty) = \left| \int dB e^{i\gamma t_e B} f(B) \right|^2 = |\text{FID}(t_e)|^2 \quad (3)$$

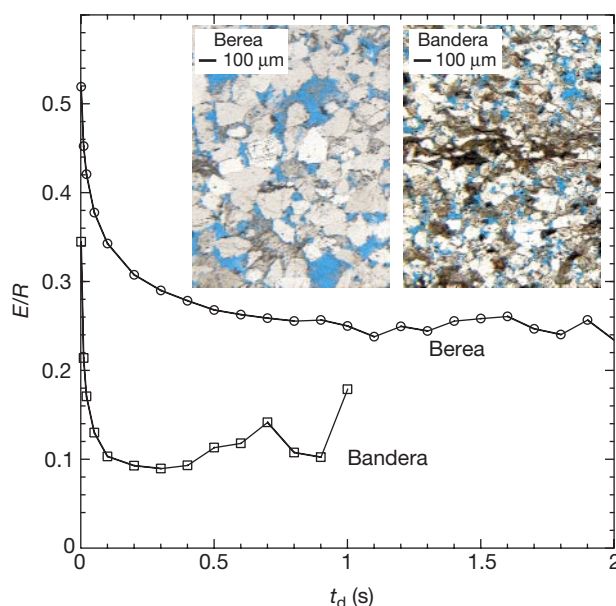


Figure 3 The DDIF ratio for two sandstone samples: Berea 100 and Bandera. Their porosities and permeabilities are: 20% and 260 mD for Berea, 21% and 19 mD for Bandera. The overall trends of the DDIF ratio are very similar except for the decay rate. The pore size L is determined to be 63 and 20 μm for the two samples, respectively. Inset, thin-section images for the two samples. A visual estimate of the pore sizes from the images is consistent with L .

independent of t_d . $E(t_c, t_d \rightarrow \infty)$ equals the square of the FID at time t_c , consistent with the direct measurement of the FID, shown in Fig. 2d. The time (t_c) it takes for the molecule to experience all B^i is related to the length scale L of the field variation, $L \approx \sqrt{2D_0 t_c}$ where D_0 is the bulk diffusion constant. These results demonstrate the utility of t_c (or L) as a direct measure of the characteristic pore dimension.

This unusual time-dependent behaviour can be obtained by a rigorous study of the magnetization diffusion and an outline of such a solution is given here. The decay of the longitudinal magnetization density during t_d is governed by the Bloch-Torrey equation^{16,17} which can be solved in terms of eigenmodes (ϕ_n) with characteristic decay time constant T_n for the n th eigenmode. In the fast diffusion limit:

$$R(t_d) \approx e^{-t_d/T_0} \quad (4)$$

$$E(t_d) \approx \alpha_0 e^{-t_d/T_0} + \sum_{n=1}^{\infty} \alpha_n e^{-t_d/T_n} \quad (5)$$

The coefficients are determined by $B_z^i(x)$: $\alpha_n = |\int dx \phi_n(x) e^{i\gamma_e B_z^i(x)}|^2 / \int \phi_n(x)^2 dx$. Brownstein and Tarr¹⁷ showed that the spatially uniform initial magnetization commonly used in relaxation measurements ensures that the decay is dominated by the lowest mode with the time constant T_0 . On the other hand, the presence of B^i changes the spatial magnetization profile at the beginning of the t_d period to something mimicking B^i that is not uniform on the pore-length

scale, which thus increases the weight of the higher modes in E . The T_n of such high modes are directly related to the pore sizes, for example, $L = n\pi \sqrt{D_0 T_n}$ for spherical pores. Thus, the enhanced faster-decaying modes in $E(t_d)$ in comparison with $R(t_d)$ may be used to determine pore size, using an inverse Laplace transform. In the case where $R(t_d)$ is approximately exponential, the ratio $E(t_d)/R(t_d)$ can be used directly to determine pore sizes via the inverse Laplace transform.

DDIF measurements for two sandstone rocks are shown in Fig. 3 with the cross-section micrographs of the pore space. The DDIF ratio shows very similar behaviour to that of the packed bead samples: with pronounced initial decay and eventual saturation. The different decay rates are indicative of the different pore size and L was evaluated to be 63 and 20 μm , respectively, consistent with the estimated pore sizes from the micrographs (insets of Fig. 3).

We have studied several carbonate rocks from the Thamama formation of the Middle East. The representative DDIF behaviour, shown in Fig. 4a, is strikingly different from that of the previous sandstone samples. The initial DDIF decay occurs over a very short time period of up to 10 ms. This decay is dominated by water diffusing within pores of the size $\sqrt{2D_0 t_c} \approx 6 \mu\text{m}$ ($D_0 \approx 2 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ at 20 °C), that is, the micropore length scale. After the initial reduction, the decay of the DDIF ratio slows down dramatically and continues to 3 s, owing to a larger dimension of the pore space, 10² μm . This data shows directly the presence of two distinct length scales associated with the micropores and the macropores of the sample. Further analysis of the DDIF ratio, using a numerical inverse Laplace transform, yields the pore-size distribution function, shown in the inset of Fig. 4a, suggesting two sub-populations of pores of 3 and 13 μm sizes, in the microporosity region corresponding to intragranular and intergranular pores. A similar range of pore sizes is also observed on the surface using electron and optical microscopy results (Fig. 1a and b).

The T_1 relaxation data for the sample is illustrated in the Fig. 4b and the T_1 distribution (inset) shows a single narrow peak. This apparently homogeneous behaviour indicates that the pore size heterogeneity, present in the DDIF experiments, must occur within the diffusion length, L_D , which is the distance a proton diffuses during a time of the order $T_1 \approx 1 \text{ s}$. An order of magnitude estimate of L_D is $\sqrt{6D_0 T_1} \approx 0.1 \text{ mm}$. In this case, the relaxation method obtains a T_1 averaged over L_D (see ref. 18 for reviews). With the combined results from DDIF and relaxation, we conclude that the multiple pore scales are intimately mixed throughout the entire rock. Thus the transport will be dominated by the intergranular pores and the macropores. Water in micropores (3 μm) is likely to be bound by the capillary pressure during production.

It is of practical importance that the signal loss in DDIF can be made very small, that is a 10–20% decay in E , in contrast to the two orders of magnitude loss of signal in conventional pulsed field gradient experiments⁷. In addition, since only radio-frequency pulses are used without pulsed field gradients, the minimum accessible diffusion time can be microseconds, extending the range of the detectable length scale to 0.1–100 μm using water. Taking advantage of the ubiquitous internal field in materials^{10–14}, this technique may find use in the *in situ* characterization of sedimentary rocks, cements and concretes, and also cells and compartmentalization in biological media. □

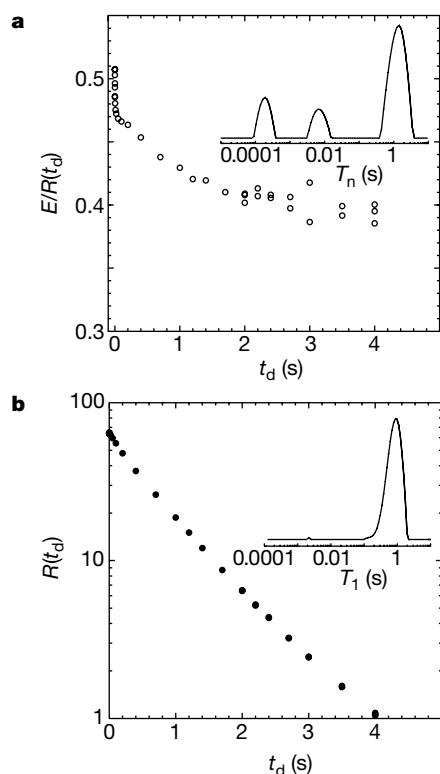


Figure 4 DDIF and relaxation measurements on a Thamama carbonate rock. The porosity and the permeability of the sample are: 23% and 4 mD. **a**, The DDIF ratio and the distributions of diffusion modes T_n (inset). The DDIF ratio versus t_d shows two distinct regimes: very fast decay up to 10 ms and a much slower decay up to 3 s, evidence of the two distinct length scales (a few μm and $\sim 100 \mu\text{m}$). The inset shows three populations of pores corresponding to the intragranular and intergranular pores and macropores. **b**, The reference signals (R) measured at the same t_d values as for **a** and the distribution of T_1 (inset). The narrow T_1 distribution indicates significant diffusion between the pores over the T_1 timescale. For both experiments, the t_d data consists of 35 points and 10 points below 20 ms.

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Cadmium toxicity among wildlife in the Colorado Rocky Mountains

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Cadmium is known to be both extremely toxic and ubiquitous in natural environments. It occurs in almost all soils, surface waters and plants^{1–3}, and it is readily mobilized by human activities such as mining⁴. As a result, cadmium has been named as a potential health threat to wildlife species⁵; however, because it exists most commonly in the environment as a trace constituent, reported incidences of cadmium toxicity are rare. Here we have measured trace metals in the food web and tissues of white-tailed ptarmigan (*Lagopus leucurus*) in Colorado. Our results suggest that cadmium toxicity may be more common among natural populations of vertebrates than has been appreciated to date and that cadmium toxicity may often go undetected or unrecognized. In addition, our research shows that ingestion of even trace quantities of cadmium can influence not only the physiology and health of individual organisms, but also the demographics and the distribution of species.

In 1969, C. E. Braun noted the existence of fragile-bone white-tailed ptarmigan (*L. leucurus*) inhabiting the Animas River watershed, near Silverton, Colorado, USA. Because this watershed drains the southwestern corner of the Colorado ore-belt⁷, and because this ore-belt region is known to contain high concentrations of a number of potentially toxic trace metals⁴, we thought that this fragile-bone condition might be caused by chronic, long-term

exposure to toxic metals, possibly exacerbated by past or present mining operations.

Between 1997 and 1999, we used multi-metals total analysis⁸ to trace metals through the ptarmigan food web within a broad section of southwest and central Colorado. Our studies showed that among all of the trace metals present in the soils and surface waters of this ore-belt region, only two—zinc and cadmium—are accumulated by ptarmigan foods in sufficient quantities to cause potential health affects in birds. Moreover, we found that cadmium is 'biomagnified' by one particular genus of plants, the willows (*Salix* spp.), a common ptarmigan food⁹. All of the species of willow, whether ground hugging or free standing, or whether growing in the alpine or in montane marshes of this region, act as biological pumps, concentrating cadmium by two orders of magnitude above background concentrations (Fig. 1). Mean cadmium concentrations in willow leaf buds and in recently grown shoots and stems were found to be $2.63 \mu\text{g g}^{-1}$ on a dry weight basis (range from 0.37 to $10.8 \mu\text{g g}^{-1}$). This concentration contrasts sharply with ptarmigan foods belonging to genera other than *Salix*, all of which were found to contain only slightly elevated cadmium concentrations ($n = 5$ species, mean $0.195 \mu\text{g g}^{-1}$; range from below detection limits to $0.35 \mu\text{g g}^{-1}$). (analysis of variance (ANOVA) t -test, $P < 0.0001$). In addition, calcium concentrations were very low ($<1\%$), and noteworthy because such low calcium levels can exacerbate cadmium uptake^{10,11}.

To determine whether comparably high cadmium concentrations existed in foods actually ingested by ptarmigan, we analysed the crop contents of 13 individual birds and found mean cadmium concentrations of $2.1 \mu\text{g g}^{-1}$. These levels were not significantly different from those found in randomly collected samples of willow leaves and leaf buds from the region.

Features of ptarmigan foraging behaviour and wintering distribution appear to affect cadmium exposure rates. First, ptarmigan have been observed in this study, and in at least one other¹², to eat more willow during winter and spring than during the summer months. When other foods, especially *Trifolium* spp., *Bistorta bistorta* and *Geum rossii*, become available (in late spring), ptarmigan switch to these less contaminated foods and, in the process, consume far less cadmium. This expansion of the diet reduces overall cadmium exposure during the summer months. Second, because females tend to over-winter at lower elevations than males¹³, and because many of these lowland areas are directly downstream from abandoned mines in Colorado, we suspected that females are exposed to higher cadmium concentrations than males.

Tissue analyses of birds of known age (determined by banding) confirmed that cadmium is accumulated (Fig. 2) at roughly the rate of $0.5 \mu\text{g}$ per day. At this rate, the average ptarmigan could accumulate toxic concentrations of kidney cadmium after only 600 days of dietary ingestion. The high net uptake rate and the long

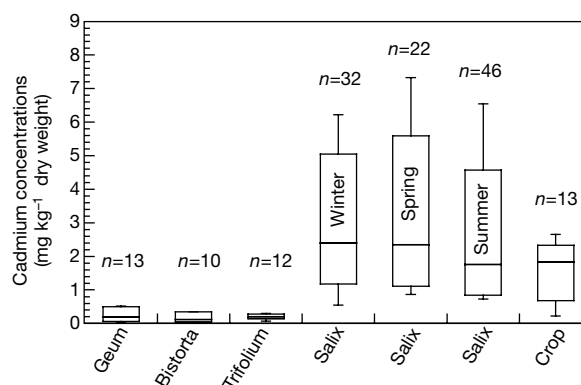


Figure 1 Cadmium concentrations in most commonly eaten ptarmigan foods (genera indicated) and in ptarmigan crops.

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biological half-life of cadmium¹⁴ create a potentially life-threatening combination for older ptarmigan in the Colorado ore-belt. Elevated kidney cadmium concentrations were found in all ore-belt birds examined (except chicks), and levels sufficient to be toxic¹⁵ were observed in 44% of adult birds. Although one-week-old chicks were observed to be almost cadmium-free (mean 1.1 ± 0.8 , range 0.6 – $2.1 \mu\text{g g}^{-1}$), older birds accumulated substantial amounts of cadmium in their kidneys (sub-adults, age 6 months, mean 21.4 ± 5.8 , range 16.99 – $27.9 \mu\text{g g}^{-1}$; young adult birds, age 9–23 months, mean 59.5 ± 29.7 , range 40 – $120 \mu\text{g g}^{-1}$; adult birds, age 24+ months, mean 99.4 ± 36.6 , range 47.4 – $188 \mu\text{g g}^{-1}$; and adult birds, age 36+ months, mean 115.5 ± 24.0 , range 98.6 – $143 \mu\text{g g}^{-1}$).

Tissue cadmium levels in white-tailed ptarmigan inhabiting the ore-belt were significantly higher than in birds inhabiting areas outside this metals-rich zone, both in the liver and kidney (Fig. 3; ANOVA t -tests, $P < 0.0001$ in both cases). While adult birds from the ore-belt were found to have toxic concentrations of kidney cadmium, adults outside the ore-belt had near-normal levels (mean $15.6 \pm 7.4 \mu\text{g g}^{-1}$).

These data suggest that cadmium accumulates continually in the ptarmigan kidney until irreversible renal tubular damage occurs¹⁶, at which time cadmium accumulations may begin to level off. We performed histopathological examinations on kidneys of 39 ptarmigan: 12 from populations with low cadmium burdens and 27 from populations with high cadmium levels. In 57% of the birds in high cadmium populations, we found renal damage, including mineral concretions surrounded by mononuclear interstitial inflammatory cell infiltrates, occasional glomeruli with granular basophilic metals and inflammatory cells, occasional dilated tubules lined with attenuated epithelium, and combinations thereof. This damage is similar to that observed in cadmium-contaminated seabirds and in cadmium-dosed starlings (*Sturnus vulgaris*)¹⁷, and is consistent with responses generated in a controlled dose-dependent study of Japanese quail (*Coturnix coturnix*)¹⁸. In our study, the most significant damage was observed in the kidneys of overwintering females. Males suffered less severe damage. Renal damage was not observed in any bird with concentrations of kidney cadmium that were lower than thought to be toxic¹⁵.

Cadmium-induced renal failure has been shown to affect calcium balance and skeletal integrity in vertebrate organisms¹⁹, but the relationship between calcium and cadmium is complex. In calcium-deficient diets, the uptake of cadmium can be facilitated^{10,11}. In diets rich in cadmium, the uptake of calcium can be retarded^{20,21}. Because ptarmigan in the ore-belt region of Colorado have diets that are poor in calcium but rich in cadmium, they appear to be doubly at risk for calcium metabolic disorders. In vertebrates in general, long-term exposure to cadmium, particularly when

combined with calcium-deficient diets, has been linked to proteinuria, bone decalcification, pseudofractures, cortical thinning and osteomalacia^{22–24}. Hens are thought to be particularly sensitive to calcium deficiencies, in part, because of their high demand for calcium during egg laying^{25,26}. We confirmed that white-tailed ptarmigan with concentrations of kidney cadmium greater than $100 \mu\text{g g}^{-1}$ frequently suffer from reduced concentrations of skeletal calcium (Fig. 4). On average, birds with toxic levels of kidney cadmium had 8–10% lower bone calcium content than either birds in the non-ore-belt reference population or birds with higher kidney cadmium levels. We propose that these data explain Braun's earlier observations of fragile bones among certain populations of Colorado white-tailed ptarmigan.

Our findings lead to four predictions concerning the life-histories of cadmium-laden ptarmigan: (1) adult survivorship will be reduced owing to age-dependent accumulation of this toxic metal, leading to renal tubular damage and skeletal thinning; (2) female survivorship will be lower than male survivorship because of their more frequent use of cadmium-rich foods in winter and because the calcium demand is greater for egg-laying females^{25,26}; (3) high rates of immigration (from nearby, less contaminated populations) will occur, owing to elevated mortality rates among poisoned populations of adult birds; and (4) lower breeding densities will be observed among contaminated populations because compensatory dispersal from surrounding populations is inhibited by the island-like distribution of their alpine habitat.

Preliminary life history data support all four predictions. On our primary cadmium-contaminated field site, yearling mortality (44%, $n = 36$, bird age 1–2 yr) was near normal for the species^{6,9}, but adult female mortality (58%, $n = 17$, bird age 3+ yr) was higher than that documented for populations outside the ore-belt⁶. As expected, mortality was highest among older, metals-contaminated ptarmigan (62%, $n = 13$, bird age 3+ yr) and was also high among adult females (71%, $n = 7$, bird age 3+ yr). Probably as a result, sex ratios were skewed in contaminated populations. The female:male ratio was generally 1:1 among sub-adult birds, but it was 3:7 in the adult population. Owing to elevated adult mortality, unusually high levels of juvenile recruitment were observed. In the 3-yr study, 40–50% of the breeding populations on all contaminated study sites were sub-adult and 80% of these birds were recruits from outside the study areas. Moreover, in a broad reversal of expected trends²⁷, sub-adults had a disproportionate influence on productivity, producing 69% of surviving young ($n = 43$, total fledglings). In our own preliminary censuses of three different habitats, breeding densities ranged from less than 1 to 8 birds per square kilometre along the

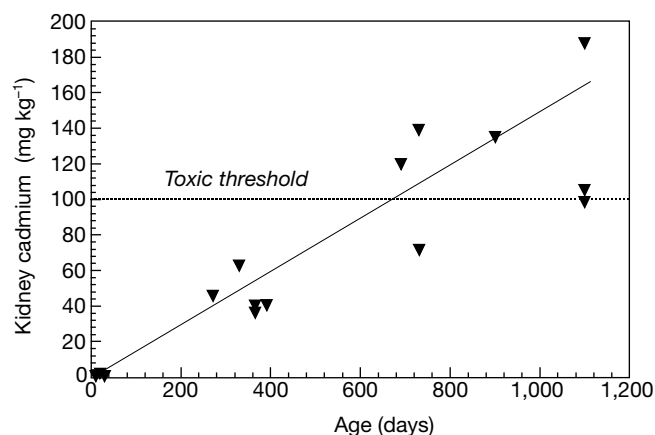


Figure 2 Kidney cadmium accumulations in Colorado white-tailed ptarmigan of known age. (Toxic threshold taken from ref. 15).

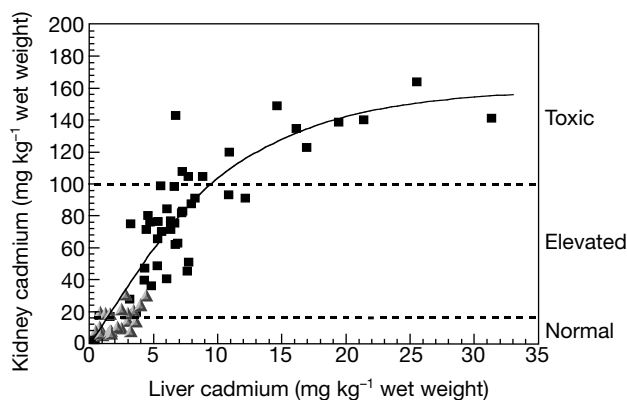


Figure 3 Kidney cadmium and liver cadmium are correlated in two populations of white-tailed ptarmigan. Among birds from the Colorado 'ore-belt' (squares), levels for both types of tissues were elevated. Moreover, the kidneys of 44% of adult ore-belt birds analysed were contaminated with critically toxic levels of cadmium. In contrast, the kidneys of adult non-ore-belt birds (triangles) contained near-normal cadmium levels.

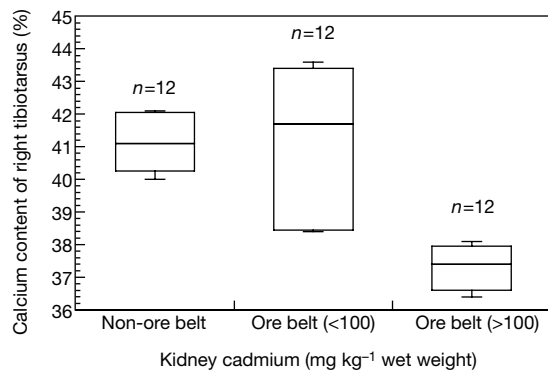


Figure 4 Bone-calcium content as a function of kidney cadmium levels in adult ptarmigan in ore-belt and non-ore-belt birds.

metals gradient. Densities were lowest in calcium-poor, acidic, metals-rich habitats and highest in calcium-poor, pH-normal, habitats with low metals levels. Our lowest-density habitat contained fewer ptarmigan than any population reported by other investigators throughout the range of the species⁹.

Cadmium exists in natural ecosystems worldwide and can be mobilized by a number of activities including mining. Willow has been shown in this study to biomagnify cadmium. Many herbivores, such as wapiti (*Cervus elaphus*), moose (*Alces alces*), mule deer (*Odocoileus hemionus*), beaver (*Castor Canadensis*) and snowshoe hare (*Lepus americanus*), eat large quantities of willow^{29,30}. Therefore, we postulate that chronic cadmium poisoning may be more widespread than shown in this study. Herbivores—particularly those that eat willow—should receive priority in future studies of cadmium toxicity in the Rocky Mountains, and elsewhere around the world where natural cadmium levels are high. We speculate that demographic and physiological effects of naturally occurring and/or anthropogenically mobilized cadmium might explain some distributional gaps or range boundaries in certain herbivorous species, and that cadmium—especially where anthropogenically mobilized—has a role in keeping some of these wildlife populations below numbers that otherwise could be supported. □

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Competition induces adaptive shifts in caste ratios of a polyembryonic wasp

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An important transition in insect life-history evolution was the shift from a solitary existence to living in groups comprising specialized castes. Caste-forming species produce some individuals that reproduce and others with worker functions that have few or no offspring¹. Morphologically specialized castes are well known in eusocial species like ants and termites¹, but castes have also evolved in less-studied groups like thrips, aphids and polyembryonic wasps^{2–5}. Because selection acts at both the individual and the colony level, ratios of investment in different castes are predicted to vary with environmental factors like competition and resources^{6–8}. However, experimental evidence for adaptive shifts in caste ratios is limited⁹ owing to the experimental difficulty of manipulating factors thought to influence caste ratios^{10–13}, and because some species produce behaviourally flexible castes that switch tasks in response to colony needs^{14,15}. Unlike other caste-forming species, the broods of polyembryonic wasps develop clonally, so that increased production of one caste probably results in decreased production of the other¹⁶. Here we show that the polyembryonic wasp *Copidosoma floridanum* alters caste

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ratios in response to interspecific competition. Our results reveal a distinct trade-off by *C. floridanum* between reproduction and defence, and show experimentally that caste ratios shift in an adaptive manner.

C. floridanum is a small (1 mm long) parasitoid wasp that oviposits one egg (female or male) or two eggs (1 female and 1 male) into the egg stage of the moth *Pseudoplusia includens*¹⁷. After parasitism, the host egg hatches and the larva develops to its final (fifth) instar. During this period, the *C. floridanum* egg proliferates clonally to form approximately 1,200 embryos¹⁶. Some embryos develop during the host's first–fourth instar into precocious larvae (Fig. 1). One or two precocious larvae are present in first instar hosts and from 2 to 10 precocious larvae develop, respectively, during the host's second to fourth instars. As a result, the number of precocious larvae progressively increases as the host caterpillar grows, to an average maximum of 30 (ref. 18). When the host reaches the fifth instar, the remaining embryos (> 1,000) develop into reproductive larvae that have smaller mandibles and a distinctly more rounded body than precocious larvae (Fig. 1). Reproductive larvae consume the host, pupate and emerge as adult wasps that disperse to locate new hosts. Precocious larvae in contrast function as a sterile soldier caste that defends its reproductive siblings from competitors^{4,5}. Precocious larvae never pupate and always die from desiccation when the host is consumed by the reproductive larvae¹⁶. We viewed the absence of task switching, ability to manipulate experimentally a restricted environment (the host), and clonal development as key attributes favouring polyembryonic wasps for studies on caste ratio evolution.

Each host attacked by *C. floridanum* represents a limited resource that can support development of only a finite number of wasps. As only reproductive larvae develop into adults, increased investment in this caste directly increases brood fitness to a maximum determined largely by the amount of host resources available for consumption¹⁸. Because precocious larvae have only soldier functions, they should increase in abundance only under heightened risk from competitors that threaten brood survival or usurp host resources. The main interspecific competitors to *C. floridanum* are other parasitic wasps like *Microplitis demolitor* that parasitize *P. includens* during the larval stage^{19,20}. These larval parasitoids possess large fighting mandibles and readily engage in combat to eliminate competitors²¹. Moreover, only one species of parasitoid usually survives when hosts are parasitized by more than one species of wasp²¹. To determine how *C. floridanum* responds to competition, we compared hosts parasitized by both *C. floridanum* and *M. demolitor* (that is, multiparasitized hosts) to hosts parasitized by each wasp alone (control). To ensure that all *C. floridanum* offspring in each host were genetically identical, broods were established from

a wasp laying a single female egg into the host egg. *M. demolitor* parasitized hosts when they were first–fourth instar larvae.

Dissection of a subsample of hosts immediately after oviposition by *M. demolitor* confirmed that this competitor always laid 1–3 eggs per host regardless of whether it contained *C. floridanum* ($n = 15$) or had not been previously parasitized ($n = 15$) ($t = 0.5$, $P > 0.1$). If these species were equal competitors, we would expect a similar proportion of multiparasitized hosts to produce *C. floridanum*, *M. demolitor*, or to die without producing any parasitoid. However, we found that 63% of multiparasitized hosts produced *C. floridanum* adults, 8% produced an *M. demolitor* adult, and 29% of hosts died without producing any parasitoid ($n = 120$) ($\chi^2 = 57.1$, d.f. = 2, $P < 0.001$). In comparison, 86% ($n = 80$) and 83% ($n = 80$) of hosts parasitized by only *C. floridanum* or *M. demolitor* respectively produced adult wasps ($\chi^2 = 0.43$; d.f. = 1, $P > 0.5$). Combined, these results indicated that *C. floridanum* was an intrinsically superior competitor to *M. demolitor*. To assess how exposure to *M. demolitor* affected caste production, multiparasitized hosts and hosts parasitized by *C. floridanum* only were dissected at the end of the fifth instar. Multiparasitized hosts contained significantly more precocious larvae ($F_{4,93} = 12.7$, $P < 0.001$) and fewer reproductive larvae ($F_{4,93} = 24.5$, $P < 0.0001$) than control hosts (Fig. 2). The shift in caste ratio and average brood size was most extreme when *M. demolitor* oviposited in first instar larvae. These hosts contained an average total of 390 *C. floridanum* larvae and 24% soldiers, whereas control hosts contained 1,034 larvae and 4% soldiers.

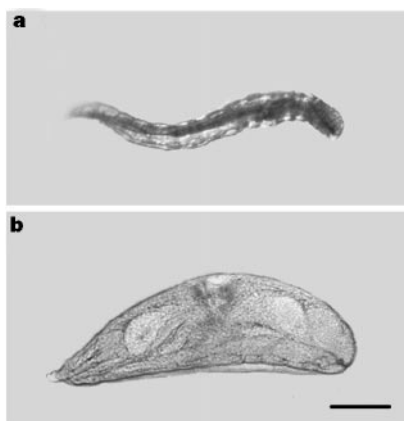


Figure 1 Light micrograph of a *C. floridanum* precocious larva (**a**) and first instar reproductive larva (**b**). Larvae were dissected from a fifth instar *P. includens* larva. The anterior (head) of each larva is oriented to the right. Scale bar, 100 μ m.

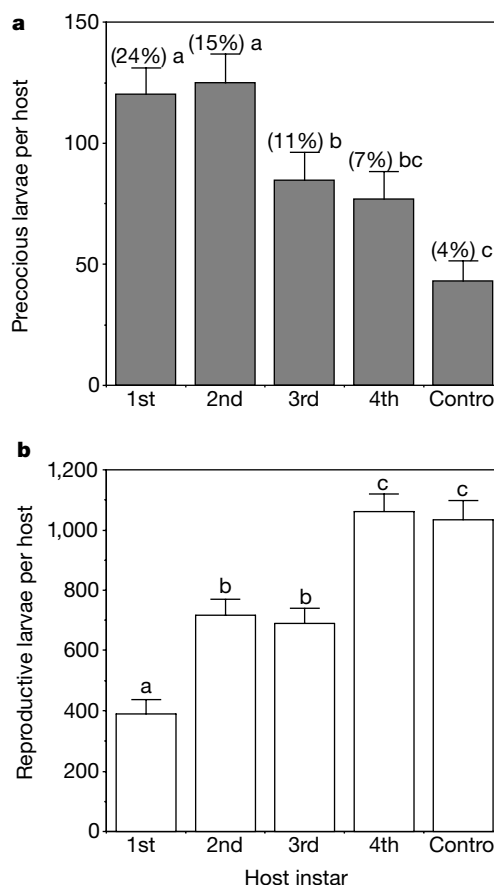


Figure 2 The effects of multiparasitism by *M. demolitor* on caste formation by *C. floridanum*. *M. demolitor* oviposited in first–fourth instar larvae, whereas control hosts contain *C. floridanum* only. All hosts were dissected in the fifth instar. **a**, The mean number of precocious larvae ($\pm$ s.e.) per host (means with the same letter were not significantly different ($P > 0.05$, Tukey–Kramer method)). The percentage of the total brood represented by precocious larvae is indicated in parentheses above each bar. **b**, The mean number of reproductive larvae per host (means with the same letter were not significantly different; $P > 0.05$, Tukey–Kramer method).

Comparison of soldier abundance on a daily basis further indicated that the number of precocious larvae per host increased significantly faster in multiparasitized hosts than in control hosts (Fig. 3).

To confirm that precocious larvae were responsible for elimination of *M. demolitor*, we observed *C. floridanum* and *M. demolitor* larvae *in vitro*. Sixty eight per cent of precocious larvae ($n = 30$) attacked *M. demolitor* during the assay period, whereas no reproductive larvae ($n = 30$) attacked *M. demolitor* during the same period ($P < 0.0001$; Fisher's Exact Test). We also determined that the abundance of soldiers critically influenced the outcome of competition by injecting from 1 to 30 precocious larvae into hosts parasitized 24 h earlier by *M. demolitor*. Hosts were dissected 7 days later and scored for the presence of a mature (final instar) *M. demolitor* larva. No *M. demolitor* survived when 15 or more precocious larvae were injected ($n = 31$), but 97% of *M. demolitor* survived when less than 5 precocious larvae were injected ($n = 30$) ($G^2 = 75.4$, $P < 0.001$, d.f. = 5). In contrast, 90% of *M. demolitor* survived in hosts injected with 1–30 reproductive larvae ($G^2 = 6.9$, $P = 0.23$, d.f. = 5).

Caste determination in insects is usually regulated by the interaction between environmental factors and endogenous developmental pathways^{1,22}. Polyembryonic wasps differ from other caste-forming species in that both castes develop from the same egg, coexist in the same environment (the host), and develop in close proximity to one another during embryogenesis²³. Proliferation and timing of morphogenesis of *C. floridanum* embryos is influenced by both host endocrine physiology and endogenous factors that result in two embryonic lineages fated to develop into precocious or reproductive larvae^{16,24,25}. The shift in caste ratio that occurs after exposure to *M. demolitor* suggests that *C. floridanum* embryos also perceive environmental cues associated with competitors. These cues could be emitted directly from the competing parasitoid larva. Larval parasitoids like *M. demolitor*, however, also inject polyDNAs in calyx fluid and venom proteins at oviposition that alter host physiology^{26,27}. These alterations could indirectly indicate the presence of a competitor. To examine the role of these cues in caste formation, third instar hosts containing *C. floridanum* were injected with 2–3 *M. demolitor* eggs, calyx fluid plus venom, or saline (control). Significantly more precocious larvae were produced in hosts injected with either eggs only ($75.9 \pm 6.1a$, $n = 20$) or calyx fluid plus venom ($86.2 \pm 5.4a$, $n = 20$) than saline ($31.0 \pm 4.4b$, $n = 20$) ($F_{2,57} = 21.2$; $P < 0.0001$; means with the same letter were not significantly different; Tukey–Kramer method). These

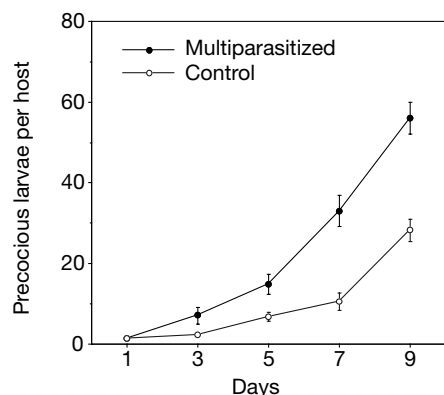


Figure 3 Mean number ($\pm$ s.e.) of precocious larvae in hosts multiparasitized by *M. demolitor* and control hosts parasitized by *C. floridanum* only. Following oviposition by *M. demolitor*, hosts were dissected every other day ($n = 8$) and the number of precocious larvae per host was counted. Overall, significantly more precocious larvae were produced in multiparasitized hosts than in control hosts ($F_{9,70} = 43.1$, $P < 0.0001$). The significant interaction between treatment and time ($F_{4,70} = 12.3$, $P < 0.0001$) indicates that the difference in soldier production between multiparasitized and control hosts increases with time.

results reaffirmed that caste ratios change in response to competition and suggest that cues from both the competing adult parasitoid and its progeny are involved in causing this shift. Increased production of precocious larvae could be due either to enhanced proliferation of embryos already fated to develop into soldiers or to a change in the fate of totipotent embryos not yet committed to one caste or the other.

The response of *C. floridanum* to interspecific competition is a dramatic example of adaptive phenotypic plasticity in a caste-forming species. As precocious larvae are incapable of reproducing, soldier fitness is determined solely by the success of their genetically identical reproductive siblings. In the absence of any competitor, caste ratios are strongly biased toward reproductive larvae, but when challenged by a competitor, caste ratios rapidly shift towards increased production of precocious larvae. This response decreases the average number of reproductive larvae produced, but enhances the probability of brood survival. Contact with one competitor may also indicate to *C. floridanum* that the abundance of interspecific competitors in the field is high, and that increased investment in defence is warranted. Similarly, colonies of the ant *Pheidole pallidula* facultatively increased soldier production when foragers regularly encountered conspecifics from neighbouring colonies⁹.

Shifts in the ratios of morphologically specialized castes are most likely to evolve when changes in an environmental factor, like competition, occur on a timescale longer than is required to produce more of a given caste (such as soldiers) to counter the threat^{6–8,22}. This situation clearly exists in *C. floridanum*, where development of precocious larvae is rapid (~ 24 h) compared to the amount of time that hosts are exposed to competitors before being consumed by reproductive larvae (15–17 days). □

Methods

P. includens and parasitoids were reared at 27 °C and a 16 h light: 8 h dark photoperiod as previously described^{18,26}. Hosts were parasitized by *C. floridanum* as 12-h-old eggs. Like most Hymenoptera, *C. floridanum* is haplodiploid with unfertilized eggs producing males and fertilized eggs producing females. All ovipositions were observed and only hosts containing a single female wasp egg were used in experiments. Identifying hosts containing a female egg was easily accomplished, because adult females exhibit different behaviours when ovipositing a fertilized and unfertilized egg¹⁷. *M. demolitor* females were allowed to oviposit once in hosts after they had become first–fourth instar larvae. Hosts were then reared until adult parasitoids emerged or were dissected to count the number and type of larvae in the haemocoel. Cohorts of hosts multiparasitized in the second instar were also dissected daily, and the number of precocious larvae present was compared to control hosts parasitized only by *C. floridanum*.

In vitro assays were conducted in 3- μ l culture wells containing TC-100 medium (JRH). *C. floridanum* and *M. demolitor* larvae were collected from hosts immediately before the experiment. A precocious or reproductive larva was placed in the culture well with a first instar *M. demolitor* larva and observed for 2 h. An attack was recorded if the *C. floridanum* larva gripped the *M. demolitor* larva with its mouthparts for more than 30 s. When this occurred, consumption of tissue was often visible. Precocious and reproductive larvae were dissected from fifth instar hosts in physiological saline and injected into third instar hosts containing *M. demolitor* using a glass needle mounted on a micromanipulator. *M. demolitor* eggs and ovariole factors (calyx fluid plus venom) were collected by dissecting female wasps in physiological saline, and removing the ovaries and venom glands²⁷. *M. demolitor* and many other larval parasitoids in the families Braconidae and Ichneumonidae carry polyDNAs which replicate in female wasps in a region of the ovary called the calyx. Virions are stored in the lumen of the oviduct, with the resulting suspension of virus and protein being called calyx fluid. In combination with venom proteins produced by the venom gland, polyDNAs are known to cause several alterations in parasitized hosts including suppression of the immune system and regulation of moulting^{26,28}. Third instar hosts containing *C. floridanum* were injected with 2–3 *M. demolitor* eggs, 0.05 wasp equivalents of calyx fluid plus venom, or saline. These dosages reflect the amount of each factor normally injected into the host by *M. demolitor* during oviposition²⁷. The number of precocious larvae was then determined by dissecting hosts in the fifth instar.

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Condition-dependent signalling of genetic variation in stalk-eyed flies

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Handicap models of sexual selection predict that male sexual ornaments have strong condition-dependent expression and this allows females to evaluate male genetic quality^{1–5}. A number of previous experiments have demonstrated heightened condition-dependence of sexual ornaments in response to environmental

stress^{6–9}. Here we show that genetic variation underlies the response to environmental stress (variable food quality) of a sexual ornament (male eye span) in the stalk-eyed fly *Cyrtodiopsis dalmanni*. Some male genotypes develop large eye span under all conditions, whereas other genotypes progressively reduce eye span as conditions deteriorate. Several non-sexual traits (female eye span, male and female wing length) also show genetic variation in condition-dependent expression, but their genetic response is entirely explained by scaling with body size. In contrast, the male sexual ornament still reveals genetic variation in the response to environmental stress after accounting for differences in body size. These results strongly support the hypothesis that female mate choice yields genetic benefits for offspring.

In the Malaysian stalk-eyed fly *C. dalmanni*, males have exaggerated eye span and females show strong mating preferences for males with greater eye span^{10,11}. Here we investigated how genotype and environment (and their interaction) affected the development of sexual and non-sexual traits. Experimental males were mated to virgin females to generate full and half-sibling families (see Methods). Eggs and larvae from each family were cultured on corn, spinach or damp cotton wool, which represent food environments of decreasing nutritional value. Emerging flies were sexed, and the male sexual trait (eye span), several comparable non-sexual traits (female eye span, male and female wing length), and body size (thorax length) were measured.

Food quality, as expected, had a large effect on absolute trait sizes (Table 1). For all traits and both sexes, spinach-fed flies were smaller than corn-fed flies ($P < 0.001$). However, the main change occurred with the shift to the poorest environment, flies that had fed on cotton wool being dramatically smaller than flies that had fed on spinach ($P < 0.001$, Fig. 1). Despite this strong condition-dependent environmental effect, there was clear evidence for genetic variation in all traits, in both sexes (Table 1). This was still the case when each environment was analysed separately (all $P < 0.05$).

Of greater interest were the interactions between effects due to food quality and effects due to genotype—that is, food quality by genotype interactions as these test whether there is a genetic basis for environmental condition dependence. For absolute male eye span, the interaction was significant (Table 1). Comparisons of the genetic variance in each environment showed that differences between genotypes increased under food stress, especially in the cotton-wool environment (Fig. 1; corn–wool $F_{29,125} = 1.937$, $P = 0.007$; spinach–wool $F_{31,113} = 1.664$, $P = 0.028$; corn–spinach $F_{31,67} = 1.462$, $P = 0.099$; tested using the Satterthwaite method, see Methods). Nonetheless genotype ranks were maintained across

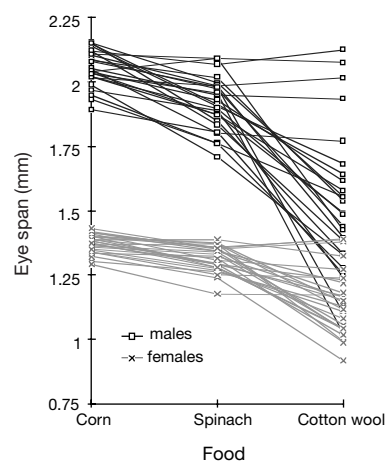


Figure 1 Genotypic responses for absolute male and female eye span in corn, spinach and cotton-wool environments. Each line represents the mean eye span of a group of full-siblings.

Table 1 Absolute trait value ANOVA

	Males†			Females		
	Eye span	Wing length	Thorax length	Eye span	Wing length	Thorax length
Food quality	85.91***	83.20***	97.73***	68.41***	76.24***	72.78***
Genotype	3.62***	3.41***	2.87***	2.39***	3.09***	2.19**
Food quality × genotype	2.05***	2.20***	1.59**	1.43*	1.82**	1.91***
N	251	241	254	279	286	286

Shown are the environmental effects due to variation in food quality, the genetic effects due to variation amongst full-sibling families, and interactions.

† F values are given for each effect; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; the degrees of freedom were d.f. = 2 (food quality), d.f. = 24–27 (genotypes) and d.f. = 48–54 (interaction).

environments (genetic correlation coefficients $\pm$ s.e.: corn–spinach $r_a = 0.660 \pm 0.106$, $P < 0.01$; corn–wool $r_a = 0.576 \pm 0.148$, $P < 0.001$; spinach–wool $r_a = 0.407 \pm 0.207$, $P < 0.001$), so a genotype which performed well in one environment tended to do well in all environments (Fig. 1). However, similar food quality by genotype interactions were present for the non-sexual traits (Table 1). Most of these (thorax and wing length in both sexes, but not female eye span) also showed increased variance in stressful environments and ranks maintained across environments (data not shown). So absolute measures of both sexual and non-sexual traits showed similar genetic components to their environmental condition-dependence.

These patterns may simply reflect scaling with body size. To discount this, we calculated relative trait values, dividing eye span and wing length by thorax length (see Methods). Relative male eye span showed the same strong environmental condition-dependence as absolute male eye span (Tables 1 and 2, similar F values). However, removing the effect of body size from the non-sexual characters reduced environmental condition-dependence of male and female wing length and female eye span (Tables 1 and 2, lower F values). These results are consistent with previous work in stalk-eyed flies demonstrating that sexual ornaments are more sensitive to environmental conditions than non-sexual traits⁹. Genetic variation

remained strong for relative trait values of both sexes, indicating that traits have genetic variation independent from body size (Table 2).

Food quality by genotype interactions showed similar changes. The strength of the food quality by genotype interaction remained unchanged for relative male eye span (Table 2). Once again genetic variance increased under food stress (Fig. 2, corn–wool $F_{32,117} = 1.981$, $P = 0.004$; spinach–wool $F_{34,99} = 1.802$, $P = 0.013$; corn–spinach $F_{41,65} = 1.140$, $P = 0.315$), and genotype ranks were maintained across environments (corn–spinach $r_a = 0.624 \pm 0.177$, $P < 0.001$; corn–wool $r_a = 0.877 \pm 0.060$, $P < 0.001$; spinach–wool $r_a = 0.206 \pm 0.262$, $P < 0.001$). In contrast, removing body size scaling from the non-sexual traits eliminated or greatly reduced any food quality by genotype interaction (Table 2). In addition, despite genetic variation being present in relative trait values of the non-sexual traits (Table 2), there was no increase in genetic variation in more stressful environments (Fig. 2, all $P > 0.05$). So for relative trait values, only male eye span showed a genetic basis to environmental condition-dependence.

Evidence that sexual ornaments have condition-dependent expression comes largely from studies using experimental manipulations of environmental stress^{6–9}. If females use sexual traits to evaluate male genetic quality these findings are paradoxical, as sensitivity to environmental variation can potentially mask genetic effects. Reduced or low heritability of sexual traits in the face of high environmental variation has been reported in several semi-natural studies of bird species^{12,13}. However, in our experiment, variation between environments, in terms of changes in food quality, magnified the differences between male genotypes (especially in the most stressful environment).

In addition, our experiment revealed the unique nature of the sexual trait, male eye span. We compared sexual and non-sexual traits, whilst controlling for general body size effects. Male eye span alone showed a genetic basis to environmental condition-dependence that was independent of body size scaling, and increased genetic variance in stressful environments. This echoes previous findings that the expression of male eye span in stalk-eyed flies is strongly informative about condition^{9,14}. Taken overall, the evidence points to a ‘good genes’ interpretation of male sexual signalling in stalk-eyed flies, as condition-dependence is predicted by handicap models of sexual selection^{1–5}. In the future, it will be important to establish whether there are similar genetic responses to environmental condition-dependence in the exaggerated sexual traits of other species. □

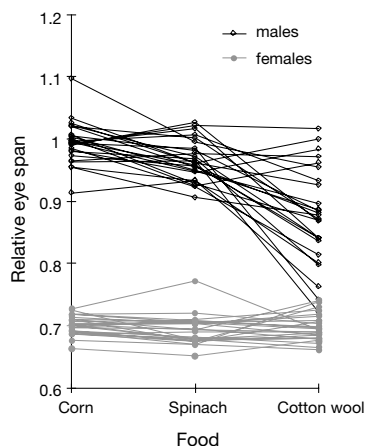


Figure 2 Genotypic responses for relative male and female eye span across the three environments. Relative values are eye span divided by thorax length for each sex. Each line represents the mean eye span of a group of full-siblings.

Table 2 Relative trait value ANOVA

	Males†		Females	
	Relative eye span	Relative wing length	Relative eye span	Relative wing length
Food quality	88.85***	10.55***	1.66 NS	29.05***
Genotype	3.82***	4.38***	2.57***	3.41***
Food quality × genotype	2.06***	0.73 NS	1.07 NS	1.454*
N	243	232	266	272

Shown are the environmental, genetic and interaction effects.

† F values are given for each effect; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, NS $P > 0.05$; the degrees of freedom were d.f. = 2 (food quality), d.f. = 24–26 (genotypes) and d.f. = 48–52 (interaction).

Methods

Fly collection

Flies used in the experiment were taken from stock populations established in 1993 from field collections near the Gombak river in Malaysia, and maintained in population cages of at least 200 individuals at 25 °C, 12 h:12 h light:dark cycle, on ground-corn medium. Virgin males ($N = 18$) were collected and mated sequentially to five virgin females, for two days each, to generate full-sibling and half-sibling families. Mated females were kept separately in large cages (20 × 20 × 40 cm) and were alternately presented with small weigh boats containing corn or spinach as a food source and egg lay medium. Weigh boats were replaced every two days and kept in individual pots to allow larval growth. Excess food (30 g per boat) was given to minimize larval competition. Females also laid eggs on the damp, cotton-wool/filter-paper cage lining. All emerging flies from these three sources were collected and frozen.

Measurements

The number of progeny was highly variable between families and environments. Twenty-nine families consisting of 12 sires mated to 2 or 3 dams provided sufficient progeny (five male and five female) from all food treatments. Stored flies were sexed before measurement of eye-stalk length (median eye-stalk bristle to the centre of the head)⁹ and wing length using a video camera mounted on a monocular microscope and the image analysis program NIH Image (Version 1.55). In addition thorax length was measured from the centre of the head to the lower edge of the thorax. All measurements were made 'blind' by a single person (T.B.). Two replicates of each measurement were taken on different days. Values were averaged over the left and right sides and the two replicates.

Statistical analysis

For each sex and each trait separately, a mixed-model ANOVA was evaluated with factors food quality (fixed), genotype (random; a genotype was defined as a full-sibling family) and the interaction¹⁵. As some families shared the same father, the analysis was repeated using hierarchical ANOVA with effects sire and dam-within-sire. Qualitatively similar relationships were found. The analysis was also carried out with pairs of environments to compute differences between environments, genetic correlation coefficients^{15,16} and their standard errors¹⁷. Genetic correlation coefficients between environments are defined as the correlation between breeding values of the same genotype in two environments¹⁶. The equality of genetic variances between two environments was tested using Satterthwaite's method¹⁵ based on the ratio $F = \frac{k_2 MS_{f,1} + k_1 MS_{e,2}}{k_1 MS_{f,2} + k_2 MS_{e,1}}$, where subscripts *f* and *e* refer to family and error mean squares, 1 and 2 to two environments, *k_i* to the number of replicates per family in environment *i* and *MS* to the mean squares. The approximate degrees of freedom for this *F* test are given in ref. 15. This ratio is constructed so that the denominator and the numerator have equal expectations when genetic variances are equal in both environments, even if error variance and sample sizes differ. For all traits and all environments, hierarchical ANOVAs with effects sire and dam-within-sire revealed no evidence of dominance or common environments as the dam component of variance was not significantly larger than the sire component^{15,16}. So our estimates of *r_a* are not inflated by dominance or effects due to common environments. The analysis was repeated using relative trait values, with thorax length used as a general measure of body size. We used relative values rather than residuals. Analysis of residuals was more complex, as traits displayed changes in their allometric slopes and intercepts across environments. A full analysis using residuals reached the same conclusions (K.F. *et al.*, manuscript in preparation). Sample sizes were not the same for different traits because occasionally specimens exhibited damage to heads or wings. Given the number of tests performed, results with borderline significance ($0.01 < P < 0.05$) are treated with caution in our discussion.

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The segment polarity network is a robust developmental module

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All insects possess homologous segments, but segment specification differs radically among insect orders. In *Drosophila*, maternal morphogens control the patterned activation of gap genes, which encode transcriptional regulators that shape the patterned expression of pair-rule genes. This patterning cascade takes place before cellularization. Pair-rule gene products subsequently 'imprint' segment polarity genes with reiterated patterns, thus defining the primordial segments. This mechanism must be greatly modified in insect groups in which many segments emerge only after cellularization¹. In beetles and parasitic wasps, for instance, pair-rule homologues are expressed in patterns consistent with roles during segmentation, but these patterns emerge within cellular fields^{2–4}. In contrast, although in locusts pair-rule homologues may not control segmentation^{5,6}, some segment polarity genes and their interactions are conserved^{3,7–10}. Perhaps segmentation is modular, with each module autonomously expressing a characteristic intrinsic behaviour in response to transient stimuli. If so, evolution could rearrange inputs to modules without changing their intrinsic behaviours. Here we suggest, using computer simulations, that the *Drosophila* segment polarity genes constitute such a module, and that this module is resistant to variations in the kinetic constants that govern its behaviour.

Gap and pair-rule gene products are nuclear proteins that form short-range gradients in the *Drosophila* syncytial blastoderm, locally modulating each other's expression through direct transcriptional control. In contrast, segment polarity genes refine and maintain their expression state through a network of cross-regulatory interactions that require cell–cell communication^{1–15}. Many segment polarity genes, unlike gap and pair-rule genes, remain active throughout development; the segment polarity network remembers the pattern imprinted upon it, then provides positional read-outs for subsequent developmental processes, including specification of neuroblasts, denticle patterns and appendage primordia. Thus, the intrinsic behaviour of the putative segment polarity module consists of stable, reiterated, asymmetric expression of its constituent genes, especially of the principal outputs *wingless* (*wg*), *hedgehog* (*hh*) and *engrailed* (*en*)¹⁵; in *Drosophila* the transient stimuli are pair-rule genes.

We used computer simulations to investigate whether the known interactions among segment polarity genes suffice to confer the properties expected of a developmental module. Given its probable conservation among diverse insects and perhaps beyond, we expected such a module to exhibit buffering against quantitative changes in gene function, and to be insensitive to the exact nature of input stimuli. We summarized (Box 1) the interactions among those segment polarity gene products that we reasoned might suffice to mimic the wild-type expression patterns of segment polarity genes (Fig. 1a). We abbreviated intermediate pathways and did not explicitly represent 'generic' components such as the transcriptional machinery. To formulate a dynamical model based on Box 1a required nearly 50 free parameters, including half-lives of messenger RNAs and proteins, binding rates, and cooperativity coefficients. The real values of these are unknown and the biologically realistic range for most parameters spans several orders of magnitude. Therefore we asked: is there any set of parameter values for

which the network model exhibits the desired behaviour, given realistic initial conditions?

Using only the solid lines in Box 1a we found no such parameter sets despite extensive efforts. Most randomly chosen parameter sets caused model components to oscillate strongly or caused some components to be expressed ubiquitously while others were repressed everywhere. Figure 1d shows the pattern most resembling the target, obtained for around 1 in 3,000 randomly chosen

parameter sets from the initial pattern in Fig. 1b. No parameter sets produced stable asymmetric patterns. We realized that if Wg is the only input to *en* and Wg is secreted symmetrically from wg-expressing cells, expression of *en* must be activated in all neighbours. Similarly, as Hh signalling activates wg in neighbouring cells, wg must be expressed on either side of *en*-expressing cells. Thus, the solid connections in Box 1a cannot suffice to explain even the most basic behaviour of the segment polarity network. There must be both active repression of *en* in cells anterior to the wg-expressing stripe and something that spatially biases the response of wg to Hh. There is good evidence in *Drosophila* for wg autoactivation¹⁶, and suggestive evidence that the Ci amino-terminal repressor fragment may inhibit *en*¹⁷. We incorporated these two possible remedies first (dashed lines, Box 1a). With these links installed there are many parameter sets that enable the model to reproduce the target behaviour, so many that they can be found easily by random sampling.

Each parameter set for which the model mimics Fig. 1a we call a 'solution' to the problem posed. Among 240,000 randomly-chosen parameter sets we found 1,192 solutions (~1 in 200). This is very frequent; as this search involved 48 parameters, on average a random choice of parameter value has roughly a 90% chance of being compatible with the desired behaviour (0.9^{48} is ~1/200). This holds even though most parameters range over several orders of magnitude. For comparison, if the model tolerated variation in the average parameter over 10% of its 100- or 1,000-fold range (a wildly optimistic expectation for a human-engineered electronic circuit), random search would find only one solution in 10^{48} samples. Figure 1e shows the stable pattern evolved for one such set of parameters from the pre-pattern in Fig. 1b. Clearly, under these conditions, the network model produces a pattern comparable to the target behaviour.

Figure 2a shows all 1,192 solutions found. Although some parameters cluster more tightly than others, none are confined to narrow sub-ranges. For each parameter, there is a solution for essentially any value. Thus, the network's ability to pass our test is intrinsic to its topology rather than to a specific quantitative tuning. There are so many diverse solutions that the notion of a globally optimal parameter set makes no biological sense. For instance, solutions for Wg diffusion rates (k_{MxferWg} , Fig. 2a) range over three orders of magnitude, from values allowing very little Wg traffic to values for which Wg diffuses rapidly across the segment.

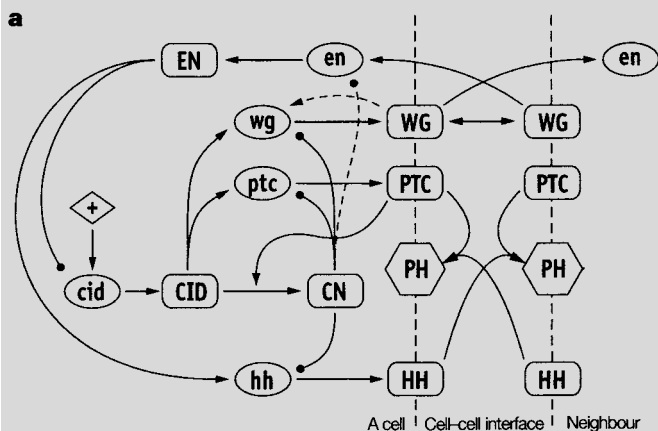
To assess sensitivity to variation in individual parameters, we took parameter sets known to produce the desired behaviour and varied one parameter while holding all others fixed. In most cases, the model tolerates tenfold or more variation in the values of individual parameters (Fig. 3). In parameter space, abrupt transitions delineate zones within which the model behaves as desired from zones of qualitatively different behaviour. The canyons of working territory are sometimes narrow, but more often broad; the model often performs equivalently despite 100- or 1,000-fold variation in the value of some of the parameters. Thus, not only does the network topology embody many different solutions, but most solutions are highly robust to variation in individual parameter values.

Aside from patterns like Fig. 1e, the model has a complex repertoire, selected by initial conditions and parameter values. Many randomly sampled parameter sets lead to ubiquitous expression of some subset of model components and global repression of others. About 1 in 700 random sets evolves the degenerate pattern in Fig. 1d for the pre-pattern in Fig. 1b. Among the most common degenerate patterns are a one-cell-wide wg stripe overlayed by a three-cell-wide *en* strip, and a stripe of *en*-expressing cells surrounded by wg-expressing cells (~1 in 70 randomly chosen parameter sets each); the former pattern results for inadequate *en* repression and the latter from excessively avid wg autoactivation. Non-degenerate patterns include *en* and wg expressed in the same one-cell-wide stripe (~1 in

Box 1

A simple continuous dynamical model of the segment polarity gene network

a, Interactions among products of the five genes in our model: WG, wingless; EN, engrailed; HH, hedgehog; CID, cubitus interruptus (whole protein); CN, repressor fragment of cubitus interruptus; PTC, patched; PH, patched-hedgehog complex. Dashed lines were added according to the insufficiencies of the bold lines alone. Ellipses, mRNAs; rectangles, proteins; arrows, positive interactions; circles, negative interactions. *ci* is basally expressed (+ in rhombus). **b**, Examples of differential equations constituting our model. These simplified dimensional-form equations govern dynamics of hedgehog mRNA, protein and the Ptc-Hh complex; several terms (hh repression by CN, transport fluxes) have been left out for clarity. See Supplementary information for further details. **c**, Simple dose-response curve governing transcriptional activation (brackets in **b**), illustrating parameterization of the model. Transcription rate saturates because of inherent limits on how fast RNA polymerase can move (T_{max}) multiplied by a gene-specific efficiency parameter (ρ_{hh}). For every monotonic regulator there is some concentration at which it has a half-maximal effect on its target (K_{ENhh}). Each such interaction may exhibit non-linearity (ν_{ENhh}). In the case of cooperative binding, ν is equivalent to a Hill coefficient.



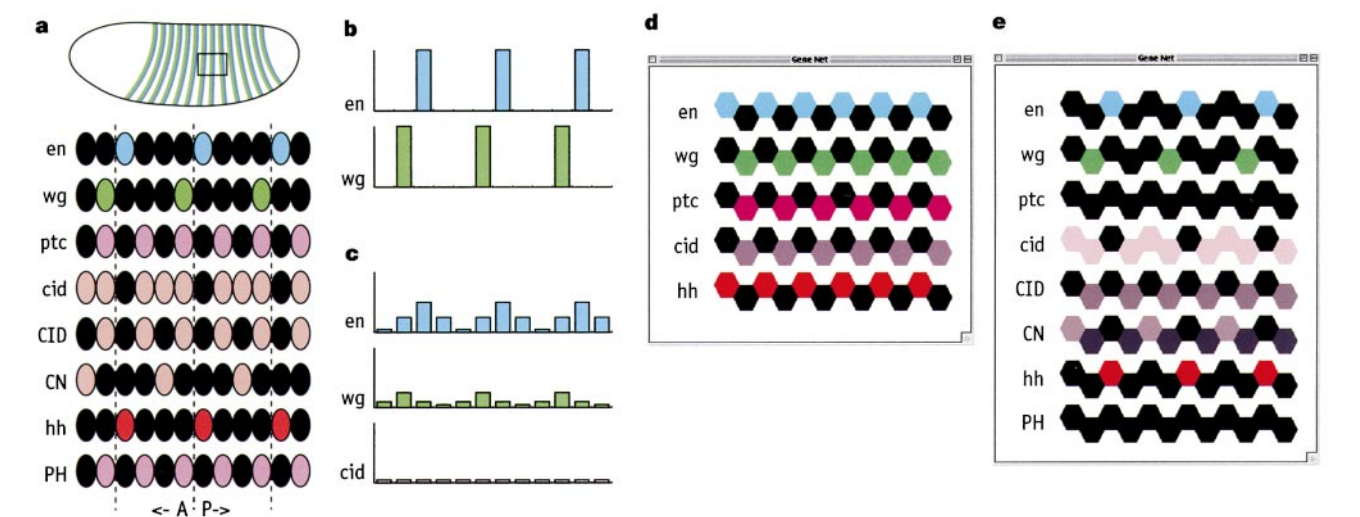


Figure 1 Segment polarity gene expression in *Drosophila*, pattern from wild type and several computer generated 'solutions'. **a**, Parasegmental boundaries divide columns of *wg*-expressing cells (green) from columns of *en*-expressing cells (blue). **b**, 'Crisp' initial conditions: *wg* activated in every fourth cell, *en* immediately posterior. *Wg* and *En* proteins are pulsed initially in the same pattern. **c**, 'Degraded' initial pattern. **d**, Best pattern

achieved with solid lines in Box 1. **e**, Pattern achieved with dashed lines installed. For clarity we show a single strip of twelve cells; we impose repeating boundaries, so all cells have six neighbours no matter how many are in the field. Adding rows or columns has no effect.

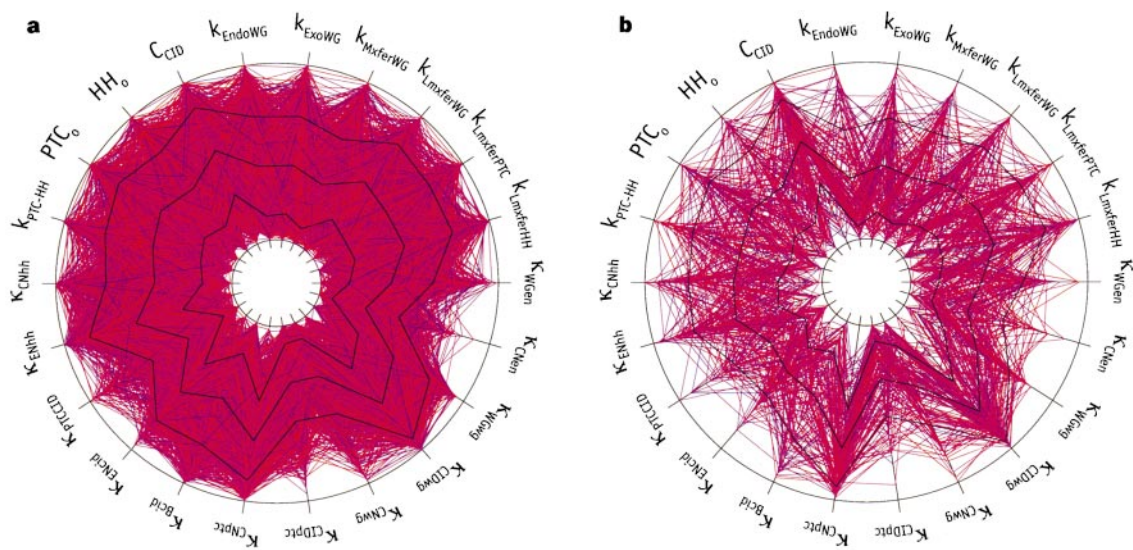


Figure 2 Graphic representation of 'solutions' obtained with crisp or degraded initial conditions. **a**, All 1,192 solutions found with crisp initial conditions. **b**, Solutions found with degraded initial conditions. Black lines plot mean and s.d. Each spoke represents the log-scale range of one parameter. Half-lives and cooperativity coefficients are omitted for clarity. Each polygon traces one parameter set as it intersects each spoke at the value of

the indicated parameter. Polygons of extreme red intensity earned best scores; those of extreme blue intensity barely passed. For k_{XXYY} , inner values represent potent regulation of *yy* by *XX*, outer values the opposite. For C_{CID} (cleavage of *Ci*), k_{PTC-HH} (*Ptc*-*Hh* binding), $k_{MxferWG}$, $k_{LmferWG}$ and similarly named (transfer processes), inner boundary means slow, outer means fast.

Table 1 Frequency of solutions as a function of initial conditions

Initial conditions	Minutes	Number of hits	Number of tries	Hit rate
Crisp: <i>wg</i> [2(h)]; <i>Wg</i> [2(h)]; <i>en</i> [3(h)]; <i>En</i> [3(h)]	200	1,192	240,000	1 in 201
Degraded: see Fig. 1c	600	149	750,000	1 in 5,000
Crisp, plus ubiquitous low-level <i>ci</i> and <i>ptc</i> : <i>ci</i> [1–4(l)]; <i>ptc</i> [1–4(l)]	200	110	41,258	1 in 375
Three-cell band of <i>ci</i> , stripe of <i>wg</i> on posterior margin: <i>wg</i> [2(h)]; <i>ci</i> [1(m),2(m),4(m)]	600	69	40,338	1 in 585
Three-cell band of <i>ptc</i> , stripe of <i>en</i> on anterior margin: <i>en</i> [3(h)]; <i>ptc</i> [1(m),3(m),4(m)]	600	127	36,196	1 in 285
Three-cell band of <i>ptc</i> , out-of-phase three-cell band of <i>ci</i> : <i>ci</i> [1(h),2(h),4(h)]; <i>ptc</i> -1(h),3(h),4(h)]	600	18	226,084	≥ 1 in 10^4
Close to target pattern: <i>wg</i> [2(h)]; <i>Wg</i> [2(h)]; <i>en</i> [3(h)]; <i>En</i> [3(h)]; <i>hh</i> [3(h)]; <i>Hh</i> [3(h)]; <i>ptc</i> [1(l),2(m),4(m)]; <i>Ptc</i> [1(l)]; <i>ci</i> [1(m),2(m),4(m)]; <i>Ci</i> [1(l),2(m),4(m)]; <i>CiN75</i> [1(m),2(l),4(l)]	200	464	21,526	1 in 46

Initial patterns coded relative to four-cell-wide segmental repeat, as in Fig. 1, with cells 1 and 2 anterior to and cells 3 and 4 posterior to the parasegmental boundary. Levels indicated as l, low (<20% of total possible); m, moderate (20–60% of total possible); h, high (60–100% of total possible). Thus, '*wg*[2(h)]' means a high level of wingless mRNA in cell 2. As indicated in the second column, all tests required acquisition of the target pattern within 200 min (for pre-patterns that contain both *wg* and *en* stripes) or 600 min plus stability over 1,000 min.

350) and *en* and *wg* expressed in overlapping three-cell-wide bands (~ 1 in 300). Although this is nowhere near a complete catalogue of the model's pattern-formation repertoire, these alternate regimes are among the 'near neighbours' of Fig. 1e in the sense that tuning parameters across the abrupt edges of canyons in Fig. 3 often yields these patterns.

We evaluated the model's sensitivity to initial conditions and discovered that the same stable pattern arises for a variety of input stimuli. The model has no 'wavelength'; that is, parameter values for which the model holds a four-cell-period repeat (Fig. 1e) enable it also to hold an equivalent three-, five- or arbitrary-period repeat pattern when triggered with a pre-pattern with corresponding spacing. We find that many solutions require only some initial bias towards expressing *wg* in one column, and *en* immediately posterior. Even for a very vaguely specified pre-pattern (Fig. 1c) we find solutions frequently (1 in 5,000)—still extremely high compared to the benchmark of 1 in 10^{48} cited above. Solutions for this case remain distributed throughout the parameter space (Fig. 2b). The model can achieve the target pattern with high frequency from initial conditions that do not include an initial pulse of *wg* or *en* (Table 1). Clearly, the model places few absolute demands on initial conditions, and it seems likely that the evolutionary process could replace inputs relatively easily.

With our model, we reconstituted *in silico* an aspect of biological behaviour from a subset of the known facts, much as a biochemist might reconstitute translation *in vitro*. Our reconstitution is far from complete. There are many additional segment polarity genes and many inputs to them. We simulated neither cell proliferation nor rearrangement, both of which affect the real network; additional

components would help to integrate patterning with morphogenesis. Many segment polarity genes function as intermediate steps between components of our model or provide outputs to downstream targets. Despite its simplicity, our model illustrates a potentially valuable benefit of the general approach. Biologists' maps of gene networks are rapidly outgrowing our ability to comprehend genetic mechanisms using human intuition alone, as shown by our initial failure. Our results reveal holes in the current understanding of segmentation: what represses *en* anterior to the *wg*-expressing stripe, and what makes Hh signalling asymmetric? We incorporated the two simplest hypotheses here, but there are hints in the literature of other candidate mechanisms. In *Drosophila* the hole-filling utility of models is limited because developmental geneticists will probably fill in the holes fast enough without help from models. For other organisms (like humans) models may complement more limited experimental opportunities.

More importantly, computer simulations allow biologists to explore emergent systems-level properties of gene networks. Boolean networks and random directed graphs have been used to capture the 'statistical mechanics' of genetic systems¹⁸. Such idealizations allowed the exploration of enormously complex systems and the discovery of generic properties of ensembles of randomly wired networks. Many have used similar methods to capture specific biologically realistic behaviours, including developmental pattern formation in *Drosophila*¹⁹. Meanwhile, the use of continuous nonlinear dynamical systems has been advocated to express cell fate determination mechanisms and the maintenance of cell states²⁰. Until recently this approach, which we take here, faced two obstacles: a paucity of facts about specific molecular mechanisms and limited computational power for solving nonlinear models. As these constraints evaporate, realistic dynamical models, based either on mass action or stochastic kinetics, will increase in usefulness. Slack foresaw that such tools would be most useful to the extent that complex genetic circuits decompose into quasi-autonomous sub-systems, that is, modules²⁰. Our work represents such a case. In another notable example, two models have been used to express the adaptive response of the bacterial chemotactic receptor, both concurring that the mechanism is highly robust^{21,22}.

The most striking systems-level property we report is the robustness to parameter variation. This is not an artefact of the wiring of our model. In work to be described elsewhere, we have analysed models that include additional links and components. Our conclusions hold for all biologically grounded variants as long as they retain the core topology shown in Box 1. Why should the segment polarity mechanism be so robust? Varying parameter values is proxy for mutations of small effect, and variation in initial conditions mimics one aspect of developmental 'noise'. We are exploring how much developmental noise embryos experience, which may explain why gene networks need buffering. Alternatively, in the evolution of segmentation there may have been pressure to neutralize mutations of small effect. We originally expected the core topology to be frail and easily perturbed, and expected to achieve robustness only by adding additional complexity; we expected the reconstitution approach to tell us which architectural features confer robustness. Confounding that expectation, the simplest model that works at all emerged complete with unexpected robustness to variation in parameters and initial conditions. □

Methods

Our model is a system of nonlinear ordinary differential equations, each characterizing the time-dependent change in concentration of one of the components of Box 1a in an indexed cell or cell face (see Supplementary Information for further details). All equations consist of either standard kinetic formulas or pseudo-steady-state approximations. Each generically includes three classes of additive term: a synthesis term, a first-order decay term, and zero or more terms representing transformation processes (heterodimerization or cleavage, for example) or flux between compartments (exocytosis or cell-to-cell traffic). We discretized diffusion according to cell faces: membrane-bound and extracellular molecules equilibrate at parameterized rates between adjacent faces of a cell, and

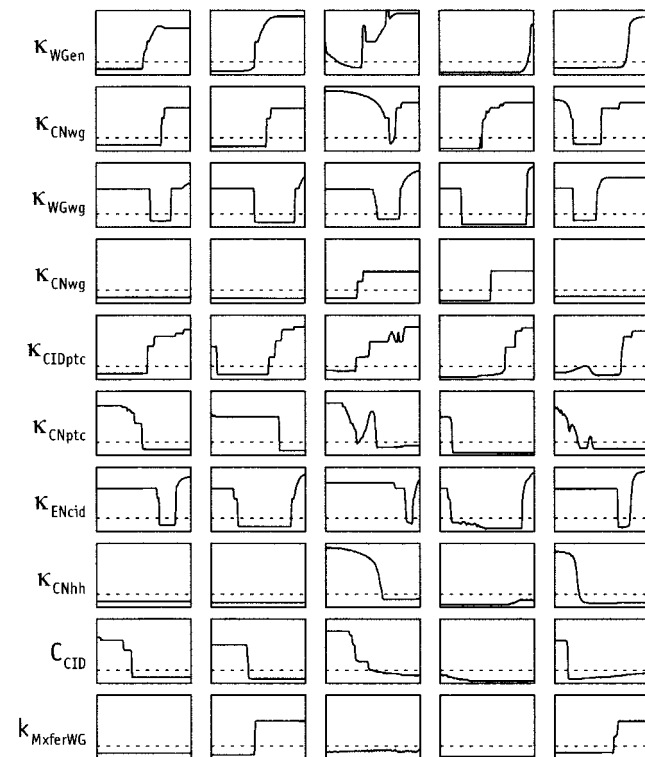


Figure 3 Sensitivity of individual solutions to varying individual parameters. Each column represents one solution, and rows are transects in which the named parameter varies while others are held fixed. The vertical axis is the goodness-of-fit score; lower scores are better matches. The dashed line indicates the boundary below which we accept the match. The horizontal axis is the parameter's log-scale range, three orders of magnitude for all. Columns one and two are typical. The third is an unusually brittle solution: κ_{CNen} and κ_{ENcid} can vary at most twofold. Column four shows the opposite extreme, and column five is a case with two working ranges for κ_{CIDptc} and κ_{CNptc} .

extracellular molecules equilibrate at parameterized rates between adjacent faces of a cell, and extracellular molecules also exchange from one cell's faces to the apposite faces of neighbouring cells.

We ran all simulations using a prototype (versions 0.62–0.66) of Ingeneue, a custom software package that we developed to construct and analyse models such as this. Ingeneue's core function is to parse a script describing a network like that in Box 1a, convert it internally into a system of differential equations, and then numerically solve the system while monitoring the time-dependent behaviour. For the network in Box 1a, each four-cell-wide by one-cell-high 'segment' repeat unit contributes 136 coupled equations that must be solved over a simulated time interval of hundreds to thousands of minutes. This calculation takes a few seconds on a desktop computer. We quantified the model's behaviour using a customized goodness-of-fit function that assigns a scalar score to each parameter set according to how well the model governed by that set matches the desired spatial pattern of gene expression within a set amount of time (typically 3 h) and holds it stably for a longer period (typically 15 h) (see Supplementary Information). Initial conditions, specified as starting concentrations, have no enforced persistence and are the only spatially heterogeneous influence. Ingeneue searches parameter space, using either random sampling or nonlinear optimization algorithms, for sets of parameters that confer on the model the desired spatial pattern formation behaviour. Ingeneue is written in Java and runs on any computer platform for which a Java Virtual Machine v.1.1.7 or better is available. Software and model files are available from the authors at <http://www.ingeneue.org>

We composed Box 1a from the literature on segmentation in *Drosophila* as follows: Wg stimulates *en* transcription in neighbouring cells^{23,24}; En promotes *hh* transcription²⁵ and represses *cr*²⁶; Hh binds to and sequesters Ptc²⁷, decreasing the rate at which Ci is processed to form a repressor, CiN75¹⁷, whereas CiN75 represses *wg* and *ptc*, full-length Ci activates these genes^{17,28–30}; either Ci or CiN75 represses *hh*²⁹; dashed lines indicate autoregulation of *wg* by an incompletely characterized pathway¹⁶, and a suggested repressive effect of CiN75 on *en*. Also, *ci* is basally expressed. Not shown but included in the model are transcytosis and cell-to-cell diffusion of Wg; as Wg transfers from cell to cell, Wg produced in a particular cell can activate targets in that cell.

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A sphingosine-1-phosphate receptor regulates cell migration during vertebrate heart development

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Coordinated cell migration is essential in many fundamental biological processes including embryonic development, organogenesis, wound healing and the immune response. During organogenesis, groups of cells are directed to specific locations within the embryo. Here we show that the zebrafish *miles apart* (*mil*) mutation^{1,2} specifically affects the migration of the heart precursors to the midline. We found that mutant cells transplanted into a wild-type embryo migrate normally and that wild-type cells in a mutant embryo fail to migrate, suggesting that *mil* may be involved in generating an environment permissive for migration. We isolated *mil* by positional cloning and show that it encodes a member of the lysosphingolipid G-protein-coupled receptor family. We also show that sphingosine-1-phosphate is a ligand for Mil, and that it activates several downstream signalling events that are not activated by the mutant alleles. These data reveal a new role for lysosphingolipids in regulating cell migration during vertebrate development and provide the first molecular clues into the fusion of the bilateral heart primordia during organogenesis of the heart.

We have taken a genetic approach to study organogenesis in vertebrates, specifically examining the morphogenetic events leading to heart formation. In all vertebrates, the myocardial progenitors involute early during gastrulation and come to occupy bilateral positions in the anterior lateral plate mesoderm (LPM). During somitogenesis, these cells undergo a second phase of migration toward the midline and fuse to form the definitive heart tube. Large-scale genetic screens in zebrafish have identified eight mutations

that disrupt this process, leading to the formation of two laterally positioned hearts (cardia bifida). One of these mutations is *miles apart* (*mil*) for which two recessive alleles exist (*mil*^{m93} and *mil*^{te273})^{1,2}. Both alleles are fully penetrant, and *mil*^{m93/te273} transheterozygous embryos display the same phenotype as homozygotes for either single mutant allele. In addition to cardia bifida, *mil* mutants display epithelial tail blisters, indicative of a defect in epithelial integrity (Fig. 1a–c). Here we report the molecular and cellular analyses of the *mil* phenotype, the positional cloning of *mil* and the biochemical and functional characterization of its gene product.

Mutations in zebrafish *hand2* cause cardia bifida and severe defects in myocardial differentiation³. To analyse myocardial differentiation in *mil* mutants, we examined the expression of *nkx2.5*, *gata6* and *cardiac myosin light chain 2* (*cmlc2*)⁴ among others. The level of gene expression and number of myocardial precursors were normal, but the location of the myocardial precursors was abnormal. By the 20-somite stage, the wild-type myocardial precursors had migrated from their anterior LPM position to the midline, whereas the mutant ones remained in their original location (Fig. 1d, e, arrows). Therefore in *mil* mutants, although the initial movements of the myocardial precursors during gastrulation appear normal, the second wave of migration from the anterior LPM to the midline is profoundly affected. Furthermore, as myocardial differentiation appears normal in *mil* mutants, these data indicate that separate signalling pathways regulate myocardial differentiation and migration.

We then examined various mesodermal derivatives to determine the extent of the migration defect. Using *hand2*, which is initially expressed throughout the LPM, *gata-1*, which is expressed in the posterior LPM, and *pax 2.1*, which is expressed in the intermediate mesoderm and pronephric ducts, we found no obvious additional defect in *mil* mutants (data not shown), indicating that the mesodermal migration defect is restricted to the myocardial precursors.

Most zebrafish cardia bifida mutations analysed to date (*oep*, *fau*, *cas* and *bon*) cause very severe endoderm defects^{5–9}, which led us to examine endoderm development in *mil* mutants. We observed a mild, partially penetrant defect in *axial*¹⁰ expression at 24 and 48 hours post-fertilization (h.p.f.). At these stages, the anterior endoderm is migrating toward the midline to form the anterior gut tube (Fig. 1f, h, arrows). In 10% of *mil*^{m93} mutants, the anterior pattern of endodermal *axial* expression is shorter in the anterioposterior axis and wider in the mediolateral axis (Fig. 1f, g). This defect becomes more pronounced with time and can result in a profound reduction in endodermal *axial* expression (Fig. 1h, i). Posterior gut endoderm appears normal in its differentiation and morphogenesis as assessed by *fkdl*, *fkdl7* and *gata6* expression (see Supplementary Information). The *mil* mutant phenotype therefore appears to reflect a

primary defect in myocardial cell migration rather than in tissue specification or differentiation.

To analyse the cell autonomy of the myocardial migration defect, we generated genetic mosaics by cell transplantation. Mutant cells in wild-type embryos migrated normally and contributed to the heart ($n = 3$), whereas wild-type cells in mutant embryos failed to migrate and remained within the bilateral myocardial structures ($n = 3$). These data indicate that the myocardial migration defect is cell non-autonomous, which means that Mil is required in a tissue other than the myocardial precursors for their migration to the midline. Mil could thus be acting in an adjacent tissue to stimulate the release of a chemotactic factor or to create an environment permissive for migration.

To gain an understanding of how myocardial cell migration is regulated during embryogenesis, we isolated *mil* by positional cloning. We used half-tetrad analysis and fine mapping to position *mil* 16.5 cM distal to Z3725 (ref. 11) on linkage group 3. Analysis of 3,168 meioses placed *mil* 1.48 cM from the embryonic α -3 *globin* gene (*zaf3*)¹². A contiguous stretch of genomic DNA was constructed from *hba*¹² using yeast artificial chromosome (YAC) and P1-derived artificial chromosome (PAC) clones (see Supplementary Information). Recombinant fine mapping determined that *mil* is located on PAC 61C4. The 100-kilobase (kb) PAC insert was used to screen a 24 h.p.f. normalized complementary DNA library. Two different clones were identified, one of which encodes a DNA methyltransferase¹³ which was not considered a good candidate for *mil*. Using a polymorphism in the 3' untranslated region of the other cDNA, we found no recombination with *mil* in 3,168 meioses. This cDNA encodes a G-protein-coupled receptor (GPCR) that shares significant identity with lysosphingolipid receptors^{14–16} (see Supplementary Information). Mil is closer in sequence to sphingosine-1-phosphate (S1P) receptors than to lysophosphatidic

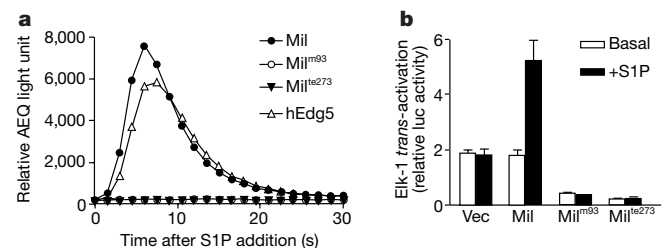


Figure 2 S1P-induced intracellular Ca²⁺ mobilization (**a**) and MAP kinase activation (**b**) requires functional Mil receptors. **a**, Addition of S1P induced intracellular Ca²⁺ mobilization in *mil* transfectants, but not in *mil*^{m93} or *mil*^{te273} transfectants. Human Edg5 was used as a positive control. **b**, Normalized Elk-1 trans-reporter gene activities are shown as mean $\pm$ s.d. of triplicate samples from one representative experiment. MAP kinase activation is seen in *mil* transfectants, but not in *mil*^{m93} or *mil*^{te273} transfectants.

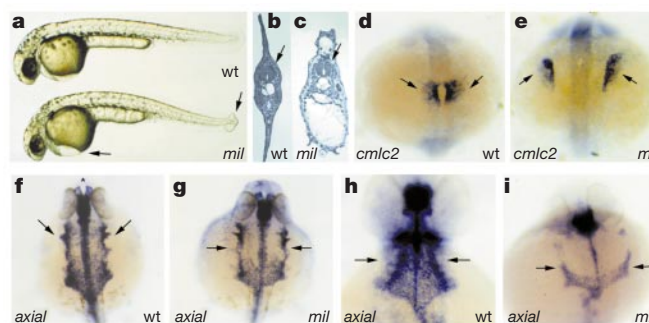


Figure 1 *mil* phenotype. **a**, Wild-type and *mil*^{m93} mutant embryos 33 h.p.f. Mutants display pericardial edema and tail blisters (arrows). **b, c**, Transverse sections through wild-type (**b**) and *mil*^{m93} mutant (**c**) tails 33 h.p.f. Arrows point to neural tube. **d, e**, Defect in myocardial migration: *cmlc2* expression in 20-somite stage (19-h.p.f.) wild-type (**d**) and

mil^{m93} mutant (**e**) embryos; arrows point to myocardial precursors. **f–i**, Defects in *axial* expression: wild-type (**f, h**) and *mil*^{m93} mutant (**g, i**) embryos 24 and 48 h.p.f. Asterisk and arrows point to expression in the ventral neuroectoderm and anterior endoderm, respectively. Dorsal views, anterior to top, are shown in **d–i**.

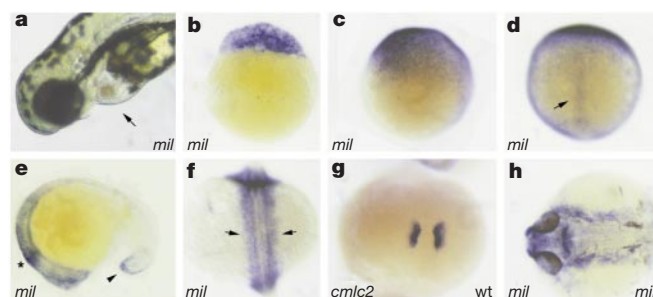


Figure 3 *mil* rescue and expression. **a**, Injecting *mil* mRNA into a *mil*^{m93} mutant can rescue heart formation as assessed by blood circulation and reduction of pericardial edema (arrow). **b–h**, *mil* expression. **b**, 128-cell embryo (2.25 h.p.f.). At tailbud (10 h.p.f.), expression is more pronounced in the anterior portion of the embryo (**c**), and embryonic axis (arrow) (**d**). **e**, At the 16-somite stage (17 h.p.f.), expression is distinct at

the midbrain/hindbrain boundary (asterisk), and the tip of the tail (arrowhead). At 18 somites (18 h.p.f.), *mil* is expressed just lateral to the midline (arrows) (**f**); *cmlc2* expression shown for comparison (**g**). At 28 h.p.f., as in all stages examined, *mil* expression is indistinguishable between wild-type and mutant embryos (**h**). Lateral views are shown in **b,c,e**, dorsal views in **d,f–h**.

acid (LPA) receptors (50% compared with 35% identity), suggesting that it could respond to S1P.

To test whether Mil could respond to S1P, we transiently transfected Jurkat T cells with *mil* (JT/Mil cells) and assayed them for S1P-mediated responses. We found that S1P treatment of JT/Mil cells mobilized intracellular calcium¹⁷ (Fig. 2a), with an effector concentration at half-maximum response (EC₅₀) value of about 10 nM (data not shown). Similar to the response of mammalian EDG-5 (ref. 18), sphingosylphosphoryl-choline treatment of JT/Mil cells also mobilized intracellular calcium. No other sphingolipid tested, including sphingosine, C2-ceramide or LPA, had any effect on JT/Mil cells (data not shown). All S1P receptors are also known to activate MAP kinase^{19,20}, and accordingly, we observed S1P-induced MAP kinase activation in JT/Mil cells, with a half-maximal effective concentration (EC₅₀) of about 10 nM (Fig. 2b). These results show that Mil is a high-affinity S1P receptor and demonstrate that bioactive lipids are important in cell migration during vertebrate development. Lipids are also critical in cell migration in invertebrates²¹. In *Drosophila*, the primordial germ cells do not migrate into areas where *wunen* is expressed²². *wunen* encodes a type 2 phosphatidic acid phosphatase for which S1P is one of a number of potential substrates²³, suggesting that regulating lipid production and degradation is important for cell migration in both vertebrates and invertebrates.

Sequencing the *m93* allele of *mil* revealed an arginine to histidine substitution at position 150 (see Supplementary Information). Arginine 150 lies at the carboxyl end of the third transmembrane domain and is part of the 'E/DRY' motif found in almost all members of the rhodopsin-like class of GPCRs. This motif is critical for receptor–G-protein coupling^{24,25}, indicating that the phenotypes seen in *mil*^{m93} mutants are due to Mil being unable to couple to downstream G proteins. Indeed, S1P-mediated signalling does not occur in JT/Mil^{m93} cells (Fig. 2a, b). The *te273* allele contains an arginine to cysteine substitution at position 167 (see Supplementary Information). Arginine 167 is found in the second intracellular loop of all EDG family members. Substituting cysteine for arginine at position 167 produces a phenotype as severe as that caused by *mil*^{m93} and results in a lack of S1P-mediated signalling in JT/Mil^{te273} cells (Fig. 2a, b).

To test further that lack of Mil function leads to the *mil* mutant phenotypes, we injected wild-type *mil* messenger RNA into embryos obtained from heterozygote intercrosses and looked for phenotypic rescue. Phenotypic analysis and genotyping of injected embryos revealed three cases (3/64) of partial phenotypic rescue (Fig. 3a). Injections of wild-type *mil* mRNA into wild-type embryos caused a variety of phenotypes, including early arrest and exogastrula formation, indicating that *mil* overexpression leads to severe developmental defects. These observations may explain, in part, why rescue was not observed more frequently. Collectively, the tight

genetic linkage, identification of severe molecular lesions and partial phenotypic rescue indicate that we have identified the *mil* gene.

The expression pattern of *mil* is complex and dynamic (Fig. 3b–h). Maternal *mil* expression is found in a diffuse pattern throughout the blastoderm (Fig. 3b), and this pattern persists through the onset of gastrulation (data not shown). More pronounced expression can be seen at tailbud stage in the anterior portion of the embryo and along the embryonic axis (Fig. 3c, d), and at the 16-somite stage in the midbrain/hindbrain boundary and the tip of the tail where blisters later develop in *mil* mutants (Fig. 3e). At the 18-somite stage, expression appears just lateral to the midline (Fig. 3f), and as the myocardial precursors migrate to the midline, their location overlaps with this domain of *mil* expression (Fig. 3f, g).

Lysosphingolipids have been shown to be important mediators of cell proliferation, differentiation and survival^{18,26–28}. Our results show that a S1P receptor is critical for cell migration and epithelial integrity during vertebrate embryogenesis. These results constitute the first molecular clues to understanding how the embryo regulates critical cellular movements during heart morphogenesis. The cell non-autonomous nature of the heart defect indicates that Mil functions outside the myocardial precursors to regulate their migration to the midline. Identification of S1P as a Mil ligand not only reveals new biological functions for lysosphingolipids, but also establishes a new class of signalling molecules during embryogenesis. □

Methods

Zebrafish strains and studies

Two alleles of *mil* (*m93* and *te273*) were used. Whole-mount *in situ* hybridization was performed as described⁴.

Cell transplantation

Donor and host embryos were obtained from *mil*^{m93} heterozygote intercrosses. Donor embryos were labelled by injection of rhodamine dextran at the 1–4-cell stage. Between 10 and 40 cells were transplanted at mid-to-late blastula stages from donor embryos to unlabelled hosts.

Linkage analysis, mapping and cloning

Diploid mutants obtained from AB/WIK hybrids were genotyped on the proximal side of the locus with *notch3*, and on the distal side with *zae3*. PAC 61C4 was sized on a pulse field gel. The coding sequence of *mil* contains no introns, so genomic DNA was cloned by polymerase chain reaction (PCR) and three independent clones sequenced for each allele. The mutations were confirmed using restriction–fragment length polymorphisms generated by the G to A substitution in *mil*^{m93} and the T to C substitution in *mil*^{te273}.

Calcium mobilization and MAP kinase measurements

Full-length *mil*, *mil*^{m93} and *mil*^{te273} cDNAs were cloned into the pEF6/V5–His mammalian expression vector (Invitrogen) and transiently co-transfected with mtAEQ/pCDNA1, which encodes the mitochondria targeting apoaquorin (Molecular Probes), into Jurkat T cells. Aequorin luminescence was measured after addition of 100 nM S1P on an EG&G Berthold microplate luminometer as described¹⁷. Transfection of empty vector alone did

not elicit any Ca^{2+} response (data not shown). To measure MAP kinase activation, pEF6-mil, pEF6-mil⁹⁵ or pEF6-mil²⁷³ were transiently co-transfected with a reporter activated by MAP kinase-stimulated Elk-1 (Stratagene) into Jurkat T cells. Normalization of transfection was performed with a pCMV-Renilla luciferase construct and the Stop&Glo reagent (Promega). After stimulation with 100 nM SIP for 4 h, activities of the Firefly and Renilla luciferases were measured in cell lysates. The C305 anti-T-cell receptor antibody gave similar Ca^{2+} and MAP kinase responses in all transfectants.

Phenotypic rescue and genotyping

For RNA injections, the mil construct was generated by PCR using wild-type cDNA and cloned into pCS2+. Injected embryos were genotyped using allele-specific restriction fragment length polymorphisms.

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A one-hit model of cell death in inherited neuronal degenerations

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In genetic disorders associated with premature neuronal death, symptoms may not appear for years or decades. This delay in clinical onset is often assumed to reflect the occurrence of age-dependent cumulative damage^{1–6}. For example, it has been suggested that oxidative stress disrupts metabolism in neurological degenerative disorders by the cumulative damage of essential macromolecules^{1,4,7}. A prediction of the cumulative damage hypothesis is that the probability of cell death will increase over time. Here we show in contrast that the kinetics of neuronal death in 12 models of photoreceptor degeneration, hippocampal neurons undergoing excitotoxic cell death⁸, a mouse model of cerebellar degeneration⁹ and Parkinson's¹⁰ and Huntington's diseases are all exponential and better explained by mathematical models in which the risk of cell death remains constant or decreases exponentially with age. These kinetics argue against the cumulative damage hypothesis; instead, the time of death of any neuron is random. Our findings are most simply accommodated by a 'one-hit' biochemical model in which mutation imposes a mutant steady state on the neuron and a single event randomly initiates cell death. This model appears to be common to many forms of neurodegeneration and has implications for therapeutic strategies.

To distinguish between the increasing risk of death associated with cumulative damage (which would generate a sigmoidal decline in cell number) and the exponential decline in cell number that results from a constant or decreasing risk of death (Fig. 1a), we used regression analysis to analyse photoreceptor neuron death in 11 animal models of inherited retinal degeneration and an experimental model of retinal detachment. These models include animals with mutations in genes encoding a range of proteins including the photosensitive pigment rhodopsin (M. M. LaVail, personal communication), the enzyme cyclic GMP phosphodiesterase^{11,12} and the structural proteins rom-1 (ref. 13) and peripherin/rds^{14,15}. In three other mutants analysed, the affected gene is unknown.

In five of these examples (Fig. 1, Table 1, and see Supplementary Information), the data fit only to mathematical models in which the probability of photoreceptor death remains constant with age. In six

others (Fig. 2, Table 1), the kinetics fit equally well to models of constant or exponentially decreasing risk of death. Consequently, in all of these animal models the increasing risk of photoreceptor death predicted by the cumulative damage hypothesis can be excluded, a possible exception being the *Rd*^{-/-} mouse in which the data (Fig. 1d) fit equally well to models of constant ($R^2 = 0.985$, $P < 0.001$) and exponentially increasing risk ($R^2 = 0.980$; $P < 0.001$). Thus, even if age-dependent cumulative damage does occur in these mutant retinas, it is not associated with an increase in the probability that the photoreceptors will die.

In agreement with these direct measurements of the kinetics of photoreceptor death in animal models, clinical assessment of photoreceptor function in patients with retinitis pigmentosa and cone-rod dystrophy has shown that both visual field loss¹⁶ and the decay of the maximum photoreceptor electroretinogram responses also observe exponential kinetics^{17,18}. Thus, the exponen-

tial cell death kinetics that we have identified in animal models appear to be shared by most, if not all, examples of inherited retinal degeneration.

To determine whether a constant or exponentially decreasing risk of neuronal death is a general phenomenon shared by other classes of neuron, we examined the kinetics of neurodegeneration in four other diseases or experimental models. Neuronal loss in the substantia nigra in Parkinson's disease¹⁰, the excitotoxic death of cultured hippocampal neurons⁸ and the loss of cerebellar granule cells in *pcd/pcd* (Purkinje cell degeneration) mice⁹ have been shown to produce an exponential decline in neuronal number with time. Our regression analyses demonstrate that only a constant risk describes the kinetics of cell death in the first two of these examples (Table 1, Fig. 3a, b). On the other hand, the loss of cerebellar granule cells subsequent to the genetically determined loss of their target Purkinje neurons in *pcd/pcd* mice⁹ (Fig. 3c) and neuronal death in a

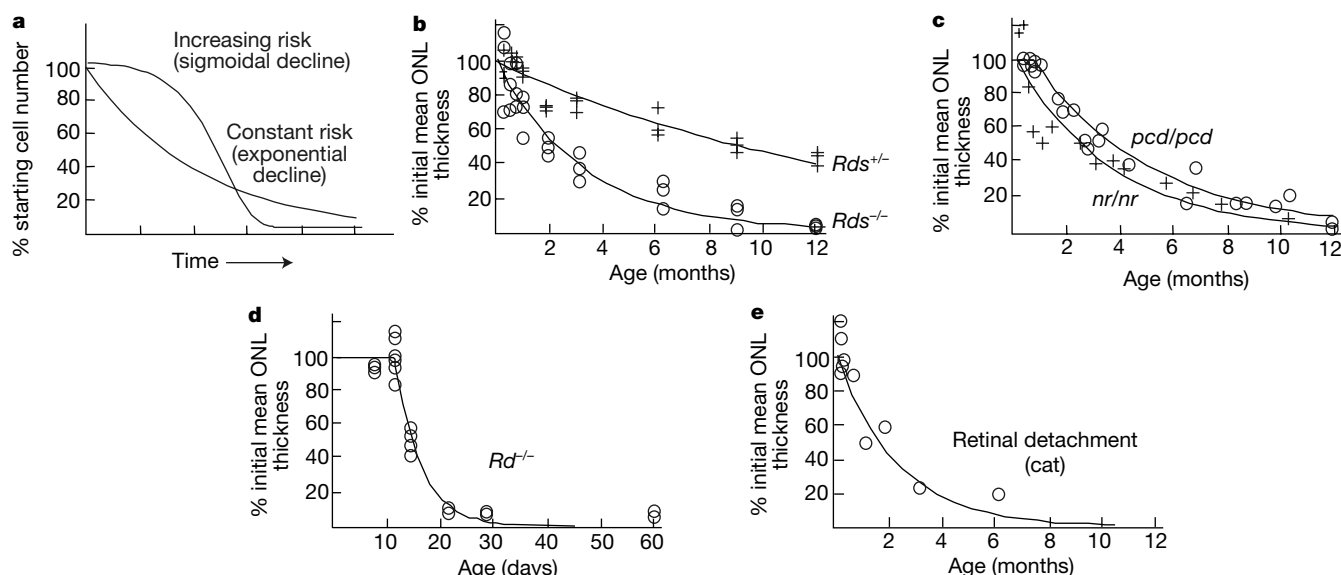


Figure 1 Animal models of inherited photoreceptor degeneration and retinal detachment, in which the kinetics of cell death are best described by a constant risk of neuronal death (see equations (1) and (3) in Methods). **a**, Constant and increasing risk of neuronal death will manifest as an exponential or sigmoidal decline in cell number, respectively. **b**, Retinal degeneration slow heterozygous (*Rds*^{+/-})¹⁵ and homozygous mice (*Rds*^{-/-})¹⁵ carrying a

null mutation in the gene encoding peripherin/rds. **c**, Nervous homozygous mice (*nr/nr*)²⁷, and Purkinje cell degeneration mice (*pcd/pcd*)²⁸. **d**, Mice homozygous for a null mutation in the gene encoding the phototransduction enzyme rod cGMP β -phosphodiesterase (*Rd*^{-/-})¹⁴. **e**, Experimental retinal detachment in the cat²⁴.

Table 1 Parameter estimates for kinetic models relating the risk of neuronal death (μ) to age

Animal model	Constant μ :			Exponentially decreasing μ :			
	μ	$\frac{dONL(t)}{dt} = -\mu \times ONL(t)$	R^2 *	μ_0	$\frac{dONL(t)}{dt} = -\mu_0 e^{-At} \times ONL(t)$	R^2 *	
<i>Rds</i> ^{+/-} mice ¹⁵	0.0170	49.4 μ m	0.992	N/A	N/A	N/A	rejected†
<i>Rds</i> ^{-/-} mice ¹⁵	0.0729	40.5 μ m	0.968	N/A	N/A	N/A	rejected†
<i>nr/nr</i> mice ²⁷	0.278	50.1 μ m	0.953	N/A	N/A	N/A	rejected†
Photoreceptors of <i>pcd/pcd</i> mice ²⁸	0.223	10.6 nuclei	0.992	N/A	N/A	N/A	rejected†
Retinal detachment (cat) ²⁴	0.00752	220 nuclei per mm	0.976	N/A	N/A	N/A	rejected†
<i>Rom1</i> ^{-/-} mice ¹³	0.0316	37.7 μ m	0.993	0.0666	0.103	41.9 μ m	0.995
<i>pd</i> (miniature schnauzer) ¹²	2.24	12.1 nuclei	0.979	3.13	1.12	13.1 nuclei	0.992
Albino (Balb/cHeA) mice ¹⁴	0.00917	51.7 μ m	0.995	0.0235	0.0485	51.7 μ m	0.997
<i>rdc-1</i> (Irish setter) ^{11,12}	0.0289	10.6 nuclei	0.957	0.0483	0.086	11.8 nuclei	0.970
<i>Rd</i> ^{-/-} / <i>Rds</i> ^{-/-} mice ¹⁴	0.123	45.4 μ m	0.992	0.208	0.0912	45.3 μ m	0.996
P23H rhodopsin-expressing transgenic rat§	0.0095	46.8 μ m	0.975	0.0172	0.00752	55.0 μ m	0.987
<i>Rd</i> ^{-/-} mice ¹⁴	0.208	42.2 μ m	0.985	N/A	N/A	N/A	rejected†
Cultured hippocampal neurons ⁸	0.0773	100% of normal	0.996	N/A	N/A	N/A	rejected†
Parkinson's disease ¹⁰	0.0858	100% of normal	0.919	N/A	N/A	N/A	rejected†
Granule cell degeneration in <i>pcd/pcd</i> mice ⁹	0.006	5,999.52 cells	0.990	0.0076	0.0018	6,000 cells	0.990
Chemically-induced rat model of Parkinson's disease ^{19‡}	0.207	100% of normal	0.967	0.537	0.310	100%	0.999

* R^2 values reflect the proportion of data variability that is explained by the model. All reported R^2 values were statistically significant ($P < 0.001$).

†Parameter estimates were not significantly different from zero.

‡Regressions performed on mean values reported in literature.

N/A, not applicable.

§M. M. LaVail, personal communication.

chemically induced rat model of Parkinson's disease¹⁹ (Fig. 3d) can both be described by either a constant or an exponentially decreasing risk of cell death (Table 1). We also measured ¹⁸F-doxyglucose uptake in the caudate nuclei of patients with Huntington's disease as an indirect measure of neuronal loss. Because each of these patients was repeatedly tested at various times after clinical onset, the glucose uptake data, predictably, is highly variable in the whole population of patients (see Supplementary Information). Consequently, analysis of neuronal degeneration kinetics in this heterogeneous population necessitated the use of repeated-measures regression for each individual (see Methods). We found that neurodegeneration in these patients is also best described by a constant risk of cell death (Fig. 3e). Thus, our identification of similar cell death kinetics in five different types of neuron indicates that a constant or decreasing risk of cell death may be common to many forms of neurodegeneration. Although neuronal death is mediated by apoptosis in all photoreceptor degenerations examined (for example, refs 7, 20), it

remains to be determined whether a constant or exponentially decreasing risk of death will be found invariably to accompany neuronal apoptosis.

Although the available data do not allow us to distinguish conclusively between constant and exponentially decreasing risk, both models indicate that the time of death of an individual neuron is random. Nevertheless, the two models have quite different pathophysiological implications. In a process involving constant risk, the time of death of an individual neuron is not only random, but also independent of the time of death of any other neuron. In this case, the kinetics of neuronal degeneration are comparable to the simple exponential decay exhibited by radioactive compounds, and the different rates of photoreceptor degeneration (Fig. 1) are determined largely or solely by the mutant genotype. In contrast, exponentially decreasing risk indicates that the chance of cell death decreases in direct proportion to the number of remaining cells. Such kinetics could result from an increase in the concentration of a

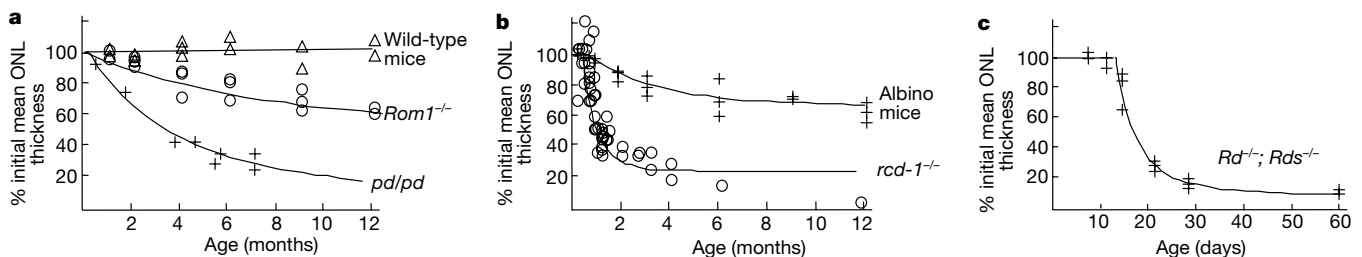


Figure 2 Animal models of inherited photoreceptor degeneration in which the kinetics of cell death are best described by an exponentially decreasing risk of death (see equations 1 and 2 in Methods). **a**, Wild-type and *Rom1*^{-/-} mice¹³, and photoreceptor dysplasia (*pd/pd*) in miniature schnauzers¹². **b**, Albino mice¹⁴ (Balb/cHeA) and rod-cell degeneration (*rcd-1*)

in Irish setters^{11,12} due to a null mutation in the rod cGMP β -phosphodiesterase gene. **c**, Mice homozygous for null mutations in both the gene encoding rod cGMP β -phosphodiesterase and the gene encoding peripherin/*rds* (*Rd*^{-/-}; *Rds*^{-/-})¹⁴.

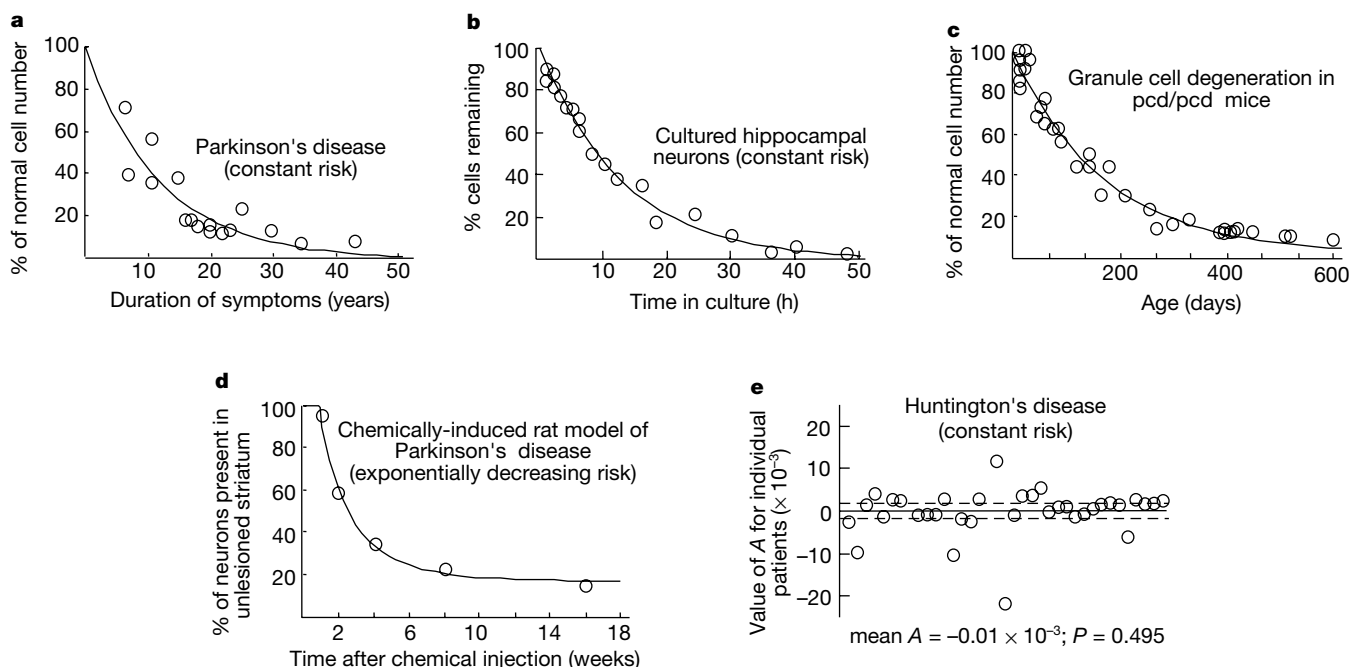


Figure 3 Examples of non-retinal neuronal death that display a constant or exponentially decreasing risk of death. **a**, The number of neurons (% of normal) in the substantia nigra of patients with Parkinson's disease as a function of symptom duration¹⁰ is best described by a constant probability of neuronal death. **b**, Cultured hippocampal neurons undergoing excitotoxic cell death after incubation with glutamate exhibit a constant risk of cell death⁹. **c**, In contrast, the secondary loss of cerebellar granule cells⁹ is described equally well by either a constant or an exponentially decreasing risk of cell death; the curve represents a constant risk. **d**, The percentage of substantia nigra neurons present in rats after injection

with the neurotoxin 6-hydroxydopamine¹⁹, a chemically induced animal model of Parkinson's disease, is best fit by an exponentially decreasing risk of neuronal death. **e**, The kinetics of metabolic decline in Huntington's disease patients are best fit by a constant risk of cell loss. Values of *A* (in the exponent of equation (5), see Methods) do not differ significantly from zero ($P = 0.495$, Student's *t*-test). Each point represents the estimated *A* for an individual patient. Solid line, mean *A* (mean $\pm$ s.d.: $-0.01 \times 10^{-3} \pm 5.3 \times 10^{-3} \text{ mM h}^{-1}$, $n = 38$ patients) across patient population; dashed lines, 95% confidence interval for the mean value of *A* (-1.7×10^{-3} , 1.7×10^{-3}).

survival factor, such as basic fibroblast growth factor or ciliary neurotrophic factor²¹, or from a decrease in the amount of a toxic factor as the population of cells declines. Consistent with the latter possibility, dying retinal progenitor cells have been shown to produce an apoptosis-inducing agent²².

An alternative interpretation for the exponential decline in risk is that the decrease is an artefact resulting from the presence of two neuronal populations, each with different but constant risks of cell death. For photoreceptor degenerations, this possibility is excluded by the fact that 97% of mouse photoreceptors are rods and only 3% are cones²³. This proportion of cones is too small to influence the overall kinetics of photoreceptor death. Furthermore, the data for all six models exhibiting a decreasing risk do not fit a simple equation incorporating two exponential functions (see Supplementary Information), which also suggests that the presence of two differentially affected cell populations is not responsible for the exponentially decreasing risk of cell death.

Any model of the mechanisms underlying inherited neuronal degenerations must account for the major features of cell death in these disorders. These features are (1) the constant or decreasing risk of neuronal death described above; (2) the genotype-dependent nature of the risk; (3) the random time of death of any cell (illustrated by the random distribution of apoptotic photoreceptors seen in the retinas of animals and humans with inherited retinal degenerations^{13,20}); and (4) the paradoxical situation in which most neurons in animals or patients with inherited neurodegenerations survive and function normally for months, years or decades, while a few genetically identical cells in the population are dying randomly.

We propose that these features can be explained if the mutant neurons are in an abnormal homeostatic state, the mutant steady state (MSS). The MSS differs little from the normal neuronal steady state (as most of the mutant cells are alive and functioning normally), except that the MSS is associated with an increased

risk of cell death. We suggest that the MSS is a response to mutation characterized by subtle but critical changes in the expression or function of relatively few 'mutant response' genes or proteins (MuRGs or MuRPs, respectively), which mediate critical pre-death reactions. If MuRP is an enzyme, for example, it may change the relative concentrations of 'pre-lethal' molecules (Fig. 4). Exit from the MSS and commitment to cell death would occur when random fluctuations in the concentrations of pre-lethal molecules exceed a threshold beyond which neuronal death is initiated. Different mutations would shift the steady state to varying degrees, so that mutations producing a transition to an MSS closer to the cell-death threshold have a greater chance of exceeding that threshold, and therefore a higher probability of causing cell death. Thus, we propose a 'one-hit' model in which the death of an individual neuron is initiated randomly in time by a single rare catastrophic event.

A similar one-hit kinetic model has been proposed and rejected as an explanation for the exponential cell death kinetics exhibited by cultured hippocampal neurons exposed transiently to excitotoxic amino acids⁸. The one-hit model was rejected in favour of a more complex mechanism involving a multistep biochemical cascade in which the overall death rate is determined by the specific rate constants for each of an unknown number of transitions within the cascade. We suggest, however, that some environmental insults place neurons in an abnormal steady state which, like the MSS, is associated with a constant increased risk of death. This environmentally induced abnormal steady state is exemplified by the effect of excitotoxic amino acids in initiating the exponential death of hippocampal neurons⁸ (Fig. 3b, Table 1) and by the effect of retinal detachment in leading to photoreceptor death²⁴ (Fig. 1e, Table 1).

Our findings have important implications for the understanding and treatment of retinal and possibly other types of neuronal degeneration. First, biochemical mechanisms proposed to underlie neuronal death must be re-examined in light of the constant or exponentially decreasing risk of cell death that we have identified. Second, identification of the MuRGs and MuRPs in different mutant neurons will indicate whether mutations impose MSSs that share common MuRGs and MuRPs, or whether each MSS is uniquely or partly defined by the specific mutant gene or mutation. In mutant neurons in which the risk of death is constant throughout life, the MSS should be the same in young and old cells with the same genotype, although additional secondary changes in gene or protein expression may occur as a consequence of cell death. Alternatively, in models in which the risk of death decreases exponentially, cell death may be associated with a changing pattern of MuRGs and MuRPs. In either case, identification of MuRGs and MuRPs in different mutants is likely to provide insight into the pathogenic events that increase the risk of cell death in the MSS. Pharmacological intervention to shift the activity of MuRGs and MuRPs towards normal levels should slow or prevent initiation of the biochemical cascade leading to cell death. Finally, the absence of cumulative damage means that the likelihood that a mutant neuron can be rescued by treatment is not diminished by age, although fewer cells will be available to rescue. Therefore, treatment at any stage of the illness is likely to confer benefit. □

Methods

Measurements and statistical analysis

We examined the kinetics of photoreceptor degeneration in animal models in which cell loss had been reported quantitatively over at least one year, or until most photoreceptors had died. The kinetics of cell loss were analysed by fitting either the outer nuclear layer (ONL) thickness or cell number data to solutions of the differential equation

$$\frac{d\text{ONL}(t)}{dt} = -\mu(t) \times \text{ONL}(t) \quad (1)$$

where $\mu(t)$ represents the risk of cell death at age t . Functions for $\mu(t)$ were substituted as

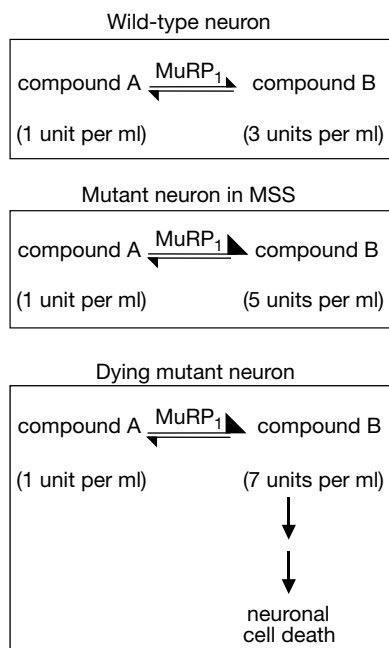


Figure 4 The exponential kinetics of cell death in inherited neuronal degenerations suggest the existence of a mutant steady state (MSS) in which the risk of cell death is increased. In wild-type neurons, a reaction, catalysed for example by the enzyme MuRP₁, is associated with a concentration of compound B of 3 units per ml. In a mutant neuron in the MSS, the MuRP₁ activity changes in response to the mutation, so that the concentration of compound B is increased to 5 units per ml. Random increases in the concentration of compound B to 7 units per ml will trigger cell death.

follows:

$$\text{exponentially decreasing risk} \quad \mu(t) = \mu_0 e^{-A(t-\text{delay})} \quad (2)$$

$$\text{constant risk} \quad \mu(t) = \mu_0 \quad (3)$$

$$\text{exponentially increasing risk} \quad \mu(t) = \mu_0 e^{A(t-\text{delay})} \quad (4)$$

where μ_0 represents the initial probability of cell death and delay represents the time before neuronal death begins. Equations for $\mu(t)$ were chosen on the basis of their ability to yield exponential and sigmoidal curves. Data fitting was performed using nonlinear regression analysis (quasi-Newton methods), a method of modelling the relationship between variables, using the functions in the Mathematica 3.0 (Wolfram Research) software package²⁵. Models were rejected if parameter estimates did not differ significantly ($P < 0.05$) from zero.

Measurements of metabolic decline in the caudates of 38 patients with Huntington's disease were obtained from PET scans as described²⁶. For each patient, average glucose uptake over the course of at least three years was fit to the solution of the differential equation:

$$\frac{d\text{ONL}(t)}{dt} = \mu e^{At} \times \text{ONL}(t) \quad (5)$$

to provide an estimate of A . $A > 0$ corresponds to increasing risk, $A < 0$ to decreasing risk, and $A = 0$ to constant risk. Estimates of A for each subject were then averaged, and a Student's t -test was used to determine whether the mean value was significantly different from zero ($P < 0.05$).

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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Genetic ablation of parathyroid glands reveals another source of parathyroid hormone

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The parathyroid glands are the only known source of circulating parathyroid hormone (PTH), which initiates an endocrine cascade that regulates serum calcium concentration¹. *Glial cells missing2* (*Gcm2*), a mouse homologue of *Drosophila Gcm*, is the only transcription factor whose expression is restricted to the parathyroid glands^{2–5}. Here we show that *Gcm2*-deficient mice lack parathyroid glands and exhibit a biological hypoparathyroidism, identifying *Gcm2* as a master regulatory gene of parathyroid gland development. Unlike *PTH receptor*-deficient mice, however, *Gcm2*-deficient mice are viable and fertile, and have only a mildly abnormal bone phenotype. Despite their lack of parathyroid glands, *Gcm2*-deficient mice have PTH serum levels identical to those of wild-type mice, as do parathyroidectomized wild-type animals. Expression and ablation studies identified the thymus, where *Gcm1*, another *Gcm* homologue, is expressed, as the additional, downregulatable source of PTH. Thus, *Gcm2* deletion uncovers an auxiliary mechanism for the regulation of calcium homeostasis in the absence of parathyroid glands. We propose that this backup mechanism may be a general feature of endocrine regulation.

Serum calcium is essential for many physiological functions including muscle contraction, blood coagulation, neuromuscular excitability and mineralization of bone, a tissue that contains 99%

of the extracellular calcium⁶. Serum calcium concentration is strictly regulated by a complex endocrine pathway of which PTH is an essential component. PTH increases serum calcium concentration by favouring calcium release from bone, stimulating renal calcium reabsorption and indirectly enhancing intestinal calcium absorption by favouring 1,25(OH)₂ vitamin D₃ synthesis¹ (Fig. 1a). No molecular determinant exclusively controlling the development of parathyroid glands has been identified. The transcription factor *Gcm2*, a mouse homologue of *Drosophila* *Gcm*²⁻⁵, is expressed exclusively in the parathyroid glands, and so may be a specific regulator of parathyroid gland development⁵. We deleted *Gcm2* using embryonic stem cell technology (Fig. 1b–d) to test this proposal.

Heterozygous mice are phenotypically normal. Crosses between these heterozygotes produced homozygous mutant *Gcm2*^{ml}/*Gcm2*^{ml} mice at the predicted mendelian frequency. Thirty per cent of the *Gcm2*-deficient mice died shortly after birth (Fig. 2a) owing to severe hypocalcaemia (as low as 3 mg dl⁻¹; Fig. 2b). The remaining *Gcm2*-deficient mice were viable, fertile and developed like their wild-type littermates. These mice had milder hypocalcaemia associated with hyperphosphataemia (Fig. 2b, c). There was also increased calcium elimination in the urine without evidence of renal failure (data not shown). These features are characteristic of hypoparathyroidism.

Eighty-five per cent of the pups generated by intercrossing *Gcm2*-deficient mice died within the first day of life (Fig. 2d). When we crossed *Gcm2*-deficient males with females heterozygous for the mutation, 78% of the *Gcm2*-deficient pups survived. When placed with foster mothers, 67% of the offspring of *Gcm2*-deficient parents

survived (Fig. 2d). These numbers are similar to those observed when intercrossing mice heterozygous for the *Gcm2* mutation (Fig. 2a). In contrast, crossing *Gcm2*-deficient females with heterozygous and homozygous males resulted in the death of 85% of the *Gcm2*-deficient pups (Fig. 2d). These data show that the lethality of the *Gcm2*-deficient pups born from *Gcm2*-deficient parents is of maternal origin, probably due to the low extracellular calcium concentration in *Gcm2*-deficient females and the decreased milk production in hypoparathyroidic rodents⁷. This was confirmed by crossing *Gcm2*-deficient males with *Gcm2*-deficient females whose serum calcium concentration had been corrected by treatment with vitamin D₃, another hypercalcaemic hormone⁸. In this case 64% of the pups survived (Fig. 2d). That the mutant phenotype reflects altered calcium and not PTH levels is also shown by the fact that there was no measurable PTH in the milk of either *Gcm2*-deficient or wild-type mice. The level of PTH related peptide (PTHrP) in both milk and serum was identical in wild-type and *Gcm2*-deficient mice (data not shown).

Regardless of the genotype of the parents, histological examination of *Gcm2*-deficient mice failed to detect any parathyroid glands (Fig. 3a). Likewise, reverse transcriptase polymerase chain reaction (RT-PCR) did not detect any *PTH*-expressing cells in the thyroid and neck of these animals (Fig. 3b and data not shown). In *Gcm2*-deficient mice, *PTH* expression is not detectable at any time point analysed (see Supplementary Information). There was no evidence of increased apoptosis in the neck region (data not shown), and no other anatomical structures were affected in *Gcm2*-deficient mice. These data identify *Gcm2* as the first specific regulator of parathyroid gland differentiation.

PTH has major effects on bone remodelling^{9,10}; we therefore analysed the skeletons of six-month-old *Gcm2*-deficient mice. The number and thickness of trabeculae were increased in these mice compared with wild-type littermates (Fig. 3c). Osteoblast and osteoclast surfaces were both reduced in *Gcm2*-deficient mice, as has been shown in WT-TPTX animals¹¹ (Fig. 3d, e), but the net result was a relative increase in bone volume without any cartilage abnormalities (Fig. 3c and data not shown). This low turnover—that is, a low cell number, high bone mass phenotype—could be rescued by continuous PTH infusion (Fig. 3c–e). The bone

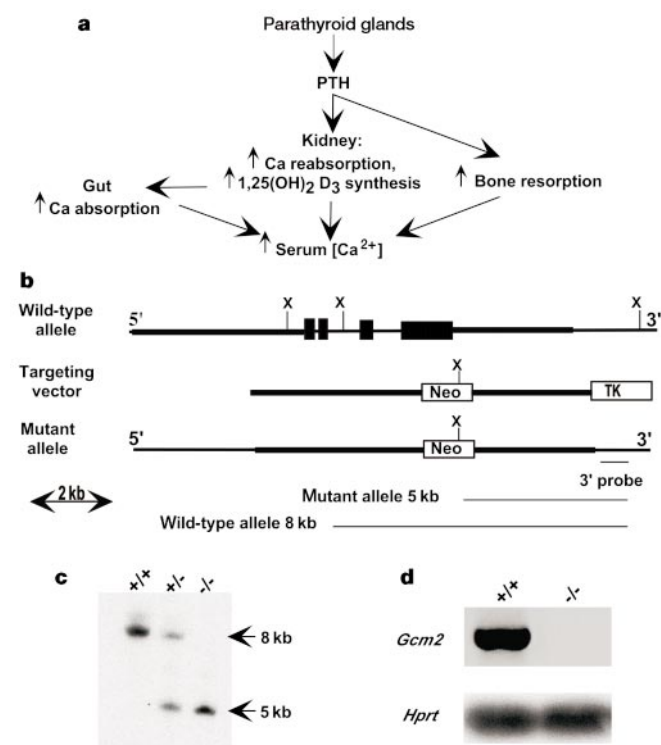


Figure 1 Regulation of calcium homeostasis and targeted disruption of *Gcm2*. **a**, PTH regulates serum calcium concentration by increasing kidney calcium reabsorption, enhancing bone resorption and increasing intestinal calcium absorption by favouring 1 α hydroxylation of 25OH vitamin D₃. **b**, Targeting strategy. Top: genomic organization of *Gcm2* with exons in black. Middle: structure of the targeting vector. Bottom: mutant allele. Probe used for Southern blot analysis. The expected sizes of wild-type and targeted alleles are indicated. X: *Xba*I. **c**, Southern blot analysis of DNA from wild-type, heterozygous and homozygous animals digested with *Xba*I. Fragment: wild-type 8 kilobases (kb) and mutant 5 kb. **d**, RT-PCR analysis of RNA isolated from the neck region.

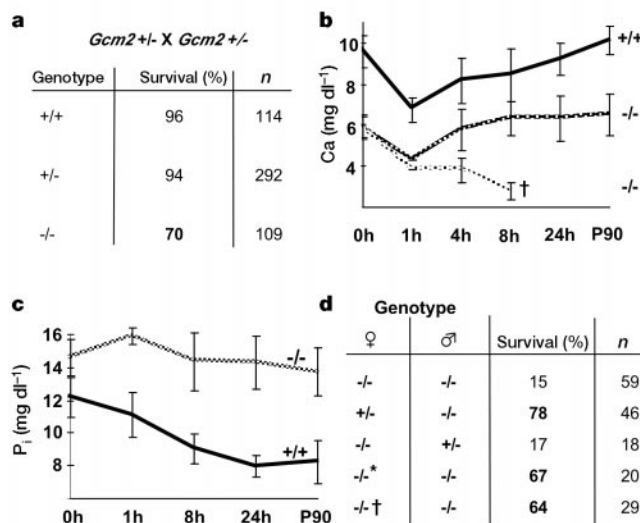


Figure 2 Fate of *Gcm2*-deficient mice and biological chemistry. **a**, 70% of *Gcm2*-deficient mice born from heterozygous matings live. **b, c**, Hypocalcaemia and hyperphosphataemia in *Gcm2*-deficient mice. **d**, Perinatal lethality of pups born from *Gcm2*-deficient females is rescued by wild-type foster mothers (–/–*) or by treatment of *Gcm2*-deficient mothers with 1,25(OH)₂ vitamin D₃ (1.2 ng per 30 g weight per day; –/–†) in the first two days of life. h: hours after birth; n: total number at birth; P: days after birth; P_i: phosphate.

phenotype of the *Gcm2*-deficient mice is mildly abnormal compared with that observed in mice and humans deficient for the PTH/PTHrP receptor^{12–14}, perhaps indicating that signal transduction through the PTH/PTHrP receptor was not completely abrogated in these mice, even though they lack parathyroid glands. This in turn suggested the existence of either a distinct, functionally redundant, ligand for the PTH/PTHrP receptor or an auxiliary source of PTH. To test the latter hypothesis we measured PTH in *Gcm2*-deficient mice using an assay that does not cross-react with PTHrP.

Serum PTH levels in *Gcm2*-deficient and wild-type mice were nearly identical and well above the limit of detection of the assay (Fig. 4a). As an internal positive control we measured PTH levels in Vitamin D receptor-deficient mice which have high serum PTH¹⁵ (Fig. 4a). In the face of a marked hypocalcaemia, the level of serum PTH in *Gcm2*-deficient mice was too low to correct the hypoparathyroidism. Continuous PTH infusion could correct the hypocalcaemia of the *Gcm2*-deficient mice (Fig. 4b). There was no compensatory increase in the serum concentration of PTHrP¹⁶ or of 1,25(OH)₂ vitamin D₃, another hypercalcaemic hormone, in *Gcm2*-deficient mice (Fig. 4c, d). That the serum concentration of 1,25(OH)₂ vitamin D₃ was normal indicates that the PTH in the serum of the *Gcm2*-deficient mice was active, as PTH is the main regulator of 1,25(OH)₂ vitamin D₃ synthesis⁸. T₃, T₄ and calcitonin serum levels were normal in *Gcm2*-deficient mice (data not shown).

To test whether the auxiliary source of PTH in *Gcm2*-deficient mice also exists in wild-type mice we measured serum calcium and PTH for various periods of time in WT-TPTX mice kept on a regular diet. The serum calcium of these animals dropped to 5 mg dl⁻¹ and their phosphorus level increased (Fig. 4e and data not

shown), demonstrating the existence of a hypoparathyroidism. But PTH was present in the serum of every WT-TPTX animal and increased over time (Fig. 4e), showing that the absence of parathyroid glands does not lead to the absence of PTH in mice.

To locate the auxiliary source of PTH we first performed RT-PCR on multiple tissues. *PTH* transcripts were found in the hypothalamus, as previously described¹⁷, but also in the thymus, the other derivative of the third pharyngeal pouch (Fig. 4f). The identity of the whole length of this transcript as *bona fide* PTH was confirmed by DNA sequencing (data not shown). *In situ* hybridization revealed a cluster of *PTH*-expressing cells underneath the thymic capsule in wild-type and *Gcm2*-deficient mice (Fig. 5a and data not shown). These thymic *PTH*-expressing cells expressed the *calcium sensing receptor* (*Casr*) gene (Fig. 5a).

Two observations indicate that the thymus is the auxiliary source of PTH. First, *Hoxa3*-deficient mice, which lack parathyroid glands and thymus but have no known hypothalamic abnormalities¹⁸, also have no detectable PTH in their sera¹⁹. This indicates that the hypothalamus does not secrete PTH into the general circulation. Second, whereas thymectomy itself did not cause lethality and only 30% of *Gcm2*-deficient and WT-TPTX mice died, 100% of wild-type mice that were thyroparathyroidectomized and thymectomized died shortly after surgery (Figs 5b, 2a). The latter experiment provides functional evidence for thymic involvement in calcium metabolism in mice lacking parathyroid glands. To determine whether this second genetic pathway of *PTH*-expressing cell differentiation could involve *Gcm1*, the other mouse homologue of

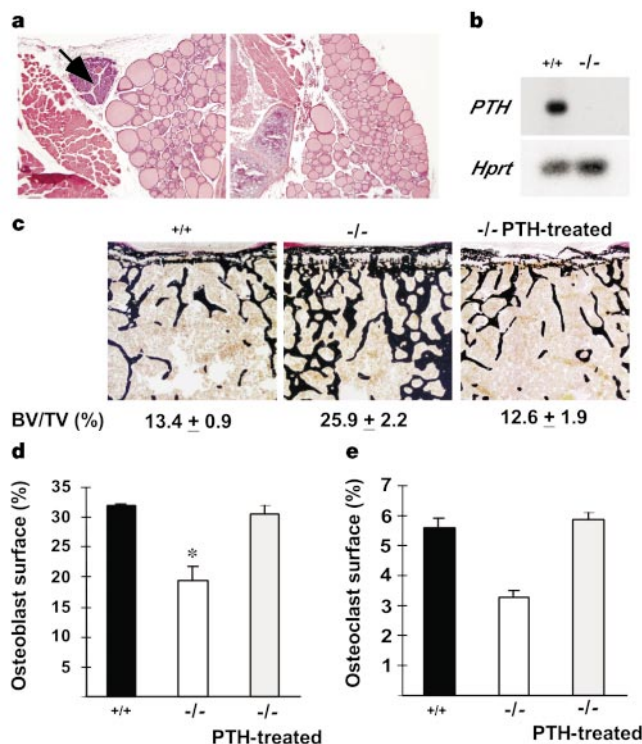


Figure 3 Absence of parathyroid glands and increased bone mass in *Gcm2*-deficient mice. **a**, Section of wild-type (left) and *Gcm2*-deficient (right) mice (arrow, parathyroid gland, present only in wild type). **b**, Absence of *PTH* signal in the neck of *Gcm2*-deficient animals (right) by RT-PCR analysis. **c**, Vertebrae of wild-type (left) and *Gcm2*-deficient mice (middle, right). Thickness of trabeculae and bone volume was increased in 6-month-old *Gcm2*-deficient mice (middle). Continuous treatment with PTH corrected the phenotype (right). **d**, **e**, Decreased osteoblast and osteoclast surface in *Gcm2*-deficient mice. The bone phenotype is rescued by PTH treatment (**c–e**). BV: bone volume; TV: tissue volume; asterisk: statistically significant difference between wild-type and *Gcm2*-deficient mice ($P < 0.05$; $n = 5$).

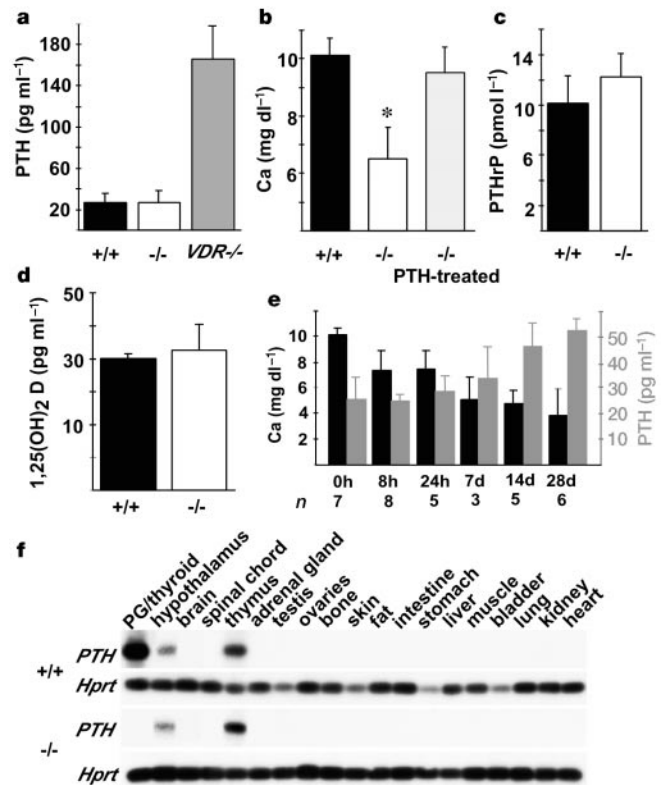


Figure 4 Detection of an auxiliary PTH source. **a**, Comparison of PTH serum levels in wild-type, *Gcm2*-deficient and *Vitamin D receptor*-deficient mice. **b**, Serum calcium level in *Gcm2*-deficient mice (white) is increased to wild-type levels (black) by continuous PTH infusion (grey). **c**, **d**, Concentration of PTHrP and 1,25(OH)₂ vitamin D₃, respectively, in serum of wild-type and *Gcm2*-deficient mice. **e**, Hypocalcaemia (black) and serum PTH (grey) in WT-TPTX mice. The analysis was done before (0 h) and 8 (8 h), 24 hours (24 h), 7 (7 d), 14 (14 d) and 28 days (28 d) after surgery. **f**, *PTH* expression in hypothalamus and thymus of wild-type (top) and *Gcm2*-deficient mice (bottom). n : number of mice analysed; asterisk: statistically significant difference ($P < 0.05$; $n = 4$); brain means brain excluding hypothalamus.

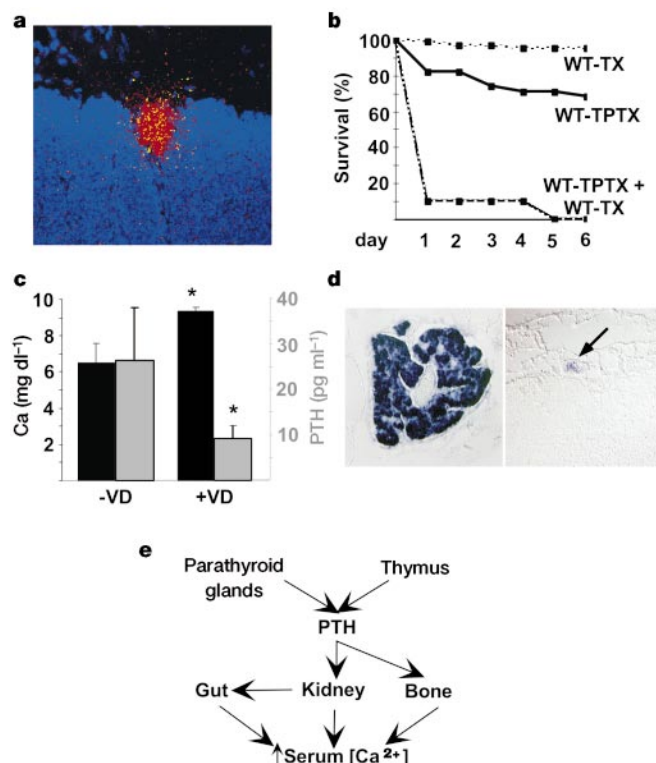


Figure 5 Physiological role of thymic PTH secretion. **a**, *In situ* hybridization revealing overlapping *PTH* (red) and *Casr* (yellow) expression in thymus. **b**, Survival of WT-TPTX, thymectomized (WT-TX) and thyroparathyroctomized and thymectomized (WT-TPTX + WT-TX) mice. **c**, Long-term treatment of *Gcm2*-deficient mice with 1,25(OH)₂ vitamin D₃

normalizes serum calcium concentration (black) and reduces serum PTH levels (grey) significantly. **d**, Size comparison of parathyroid glands (left) and the thymic source of PTH (right). **e**, Revised model of calcium homeostasis indicating the auxiliary role of the thymus. Asterisk: statistically significant difference ($P < 0.05$; $n = 4$).

Drosophila Gcm (ref. 4), we looked for its expression in thymus and parathyroid glands. *Gcm1* expression colocalized with *PTH* expression, indicating that *Gcm1* could contribute to the differentiation of *PTH*-expressing cells in thymus (see Supplementary Information). We failed to detect *Gcm1* expression in parathyroid glands (data not shown).

The absence of a high PTH serum level in the *Gcm2*-deficient and WT-TPTX animals despite hypocalcaemia suggested that the thymic PTH source already secreted its maximum amount of PTH and/or that it could not be regulated properly. Long-term treatment with vitamin D₃, a hormone that increases serum calcium concentration and decreases PTH synthesis in wild-type animals²⁰, had the same effects in *Gcm2*-deficient mice (Fig. 5c), indicating that secretion of the thymic PTH could be downregulated by one physiological regulator. In contrast, treatment of *Gcm2*-deficient mice with sodium phosphate, which decreases serum calcium and increases PTH in wild-type mice²¹, failed to do so in *Gcm2*-deficient mice (see Supplementary Information). The absence of upregulation of PTH secretion by the thymic source in response to low serum calcium indicates that these cells, which do express *Casr*, secrete their maximum amount of PTH in the absence of parathyroid glands. This would be consistent with the very small size of this source compared with the size of the parathyroid glands (Fig. 5d).

From a developmental perspective this study identifies *Gcm2* as the first gene, to our knowledge, specifically to control the differentiation of cells of the third pharyngeal pouch into parathyroid glands. Moreover, *Gcm2* deletion uncovers the existence of an auxiliary genetic pathway, possibly involving another *Gcm* protein, that can achieve differentiation of PTH-producing cells in the thymus. From a physiological point of view, our results uncover a general feature of serum calcium regulation that has not been appreciated before (Fig. 5e). The existence of an auxiliary source

of PTH accounts for the relatively mild biochemical and bone phenotype of the *Gcm2*-deficient mice. It also provides a genetic explanation for the previously suspected regulation of calcium and phosphate metabolism independently of the parathyroid glands and for the high incidence of PTH-producing tumours in thymus²². On the basis of our results we predict that a *PTH*-deficient mouse will have a more severe phenotype than the *Gcm2*-deficient mouse.

Beyond calcium regulation itself, the existence of an auxiliary source of PTH bears some resemblance to other endocrine-failure situations. In ovarian failure, for instance, the production of oestrogens by other tissues does not prevent menopause²³. These two examples among others suggest that auxiliary sources of hormone are a common safety feature of endocrine regulation, implying that the phenotype of an endocrine organ failure will in most cases be less severe than the phenotype caused by the absence of the corresponding hormone. □

Methods

Targeting strategy

We constructed a targeting vector containing 8 kilobases of homology to the region of the *Gcm2* locus, a neo cassette for positive selection and a thymidine kinase cassette (TK) for negative selection²⁴. The neo cassette replaces all four exons⁵. The vector was electroporated into AB-1 mouse embryonic stem (ES) cells and the ES clones were selected in the presence of G418 and FIAU²⁴. DNA prepared from resistant clones was digested with *Xba*I and screened by Southern blot using a 0.8-kb *Xba*I–*Hind*III fragment as a probe. Correctly identified targeted clones were injected into C57BL/6 blastocysts and germline transmission was obtained as described²⁴.

RT-PCR

DnaseI-treated RNA isolated using Trizol (Gibco) was used for reverse transcription. PCR involved annealing at 60 °C for *Gcm1*, 56 °C for *Gcm2* or 58 °C for *Hprt* and 67 °C for *PTH* for 35 cycles or 25 cycles for *Hprt*. The following primers were used: *Gcm1* RT1F: 5'-GCACGAATCAATG GAAGTGGACGACTTTG-3'; *Gcm1* RT1B: 5'-TAGCTGCTCAGATCCACAGA-3'; *Gcm2* F7: 5'-CGGAATTCCTATTCTCGCTCTTAACCTTTGCC-3'; *Gcm2* B7: 5'-GCTCTAGACCAGCAAGTTACAGGTCTGAGCTTC-3'; *HPRT*5: 5'-

GTTGAGAGATC ATCTCCACC-3'; HPRT3: 5'-AGCGATGATGAACCAGGTTA-3'; PTHF-RT: 5' ATGA TGTCTGCAAGCACCATTGGCT-3'; PTHB-RT: 5'-CGGTCTAGAAT ACGTCAGCATT TA-3'.

Biological chemistry

Serum calcium and inorganic phosphorus concentrations were determined colorimetrically (Sigma). PTH concentration was determined using a two-sided immunoradiometric assay specific for rat PTH (1–34) (Nichols Institute). The detection limit is 2 pg ml⁻¹. Continuous subcutaneous treatment of adult mice was done by delivery of 2.8 ng human PTH(1–34) per g weight per h (Bachem) in buffer (1mM HCl, 0.15M NaCl; 2% cysteine, 10% heat inactivated BSA) for 6 days and subsequent treatment with 2.2 ng human PTH(1–34) per g weight per h for 8 days using miniosmotic alzet pumps (Alza). Controls were treated with buffer. PTHrP was analysed using a radioimmunoassay detecting the amino-terminal portion of the human protein²⁵.

In situ hybridization and histology

In situ hybridization was carried out as described²⁶ using digoxigenin-labelled riboprobes for rat PTH¹⁷, rat *Casr*²⁷, mouse *Pax9* (ref. 26) and mouse *Gcm1*. The *Gcm1* fragment (base pairs 838–1460) was subcloned into bluescript, linearized with *XbaI* and transcribed with T3. Bone specimens were processed as described²⁸

TPTX and thymectomy

Thyroparathyroidectomy and thymectomy was performed on six-week-old animals (Taconic). All animals were maintained on a regular diet *ad libitum* without additional calcium supplementation.

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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A cholesteryl ester transfer protein inhibitor attenuates atherosclerosis in rabbits

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Cholesteryl ester transfer protein (CETP) is a plasma protein that mediates the exchange of cholesteryl ester in high-density lipoprotein (HDL) for triglyceride in very low density lipoprotein (VLDL)^{1,2}. This process decreases the level of anti-atherogenic HDL cholesterol and increases pro-atherogenic VLDL and low density lipoprotein (LDL) cholesterol, so CETP is potentially atherogenic^{3–9}. On the other hand, CETP could also be anti-atherogenic^{10–14}, because it participates in reverse cholesterol transport (transfer of cholesterol from peripheral cells through the plasma to the liver)¹⁵. Because the role of CETP in atherosclerosis remains unclear, we have attempted to develop a potent and specific CETP inhibitor. Here we describe CETP inhibitors that form a disulphide bond with CETP, and present one such inhibitor (JTT-705) that increases HDL cholesterol, decreases non-HDL cholesterol and inhibits the progression of atherosclerosis in rabbits. Our findings indicate that CETP may be atherogenic *in vivo* and that JTT-705 may be a potential anti-atherogenic drug.

After we found that disulphide compound 1 (Table 1) inhibited CETP-mediated transfer of cholesteryl ester from HDL to VLDL and LDL in human plasma by 50% at a concentration of 500 µM, we systematically varied the partial structures (alkyl, amide and disulphide moieties) of compound 1 to obtain a highly potent CETP inhibitor. We have summarized the results of these structure-activity studies in Table 1. The alkyl moiety markedly increased potency, indicating that this moiety may promote specific binding to CETP. As the reversed amide 5 and sulphide 6 showed loss of activity, both the amide group and the disulphide group are

probably essential for inhibition of CETP. On the other hand, thiol **7** also possessed inhibitory activity, indicating that the thiol group may function like the disulphide group and that a dimeric structure was not necessary. The thioester (JTT-705), which was readily hydrolysed to the active thiol, also inhibited CETP. These data show that the structural requirements for the inhibitors are strict, indicating that the optimum compounds (such as **4**, **7** and JTT-705) may be structurally specific inhibitors of CETP.

Because a common property of both disulphides and thiols is the ability to form a disulphide bond with another thiol group, we hypothesized that formation of such a bond between the test compounds and a cysteine residue of CETP may be related to the inhibitory mechanism of these compounds. We therefore constructed CETP mutants in which the cysteine at residue 1 (C1S), 13 (C13S) or 131 (C131S) was replaced by serine. JTT-705 could inhibit the C1S and C131S mutants, but could not inhibit the C13S mutant (Fig. 1). The cysteine at residue 13 of CETP thus seemed to be essential for the inhibitory activity of JTT-705. The S-methyl compound **8** could not form disulphide bonds and showed loss of activity, as expected, supporting our proposed mechanism of action.

As the disulphides were not orally bioavailable and the thiols were unstable, we used orally bioavailable thioesters (JTT-705) for *in vivo* assays. The thioester JTT-705 produced 95% inhibition of CETP activity at an oral dose of 30 mg kg⁻¹ in Japanese white rabbits. Although several studies have shown that certain cysteine-modifying agents inhibit CETP under serum-free conditions^{16,17}, there have been no reports on the activity of such compounds in the plasma or

in vivo. As such cysteine-modifying agents are highly reactive, nonspecific reactions with endogenous cysteine-containing peptides (such as glutathione) or proteins may decrease their inhibition of CETP. On the other hand, JTT-705 was specific enough to inhibit CETP *in vivo*. To our knowledge, this is the first report of *in vivo* inhibition of CETP activity by a cysteine-modifying compound.

Although we did not identify complexes of CETP and JTT-705 in

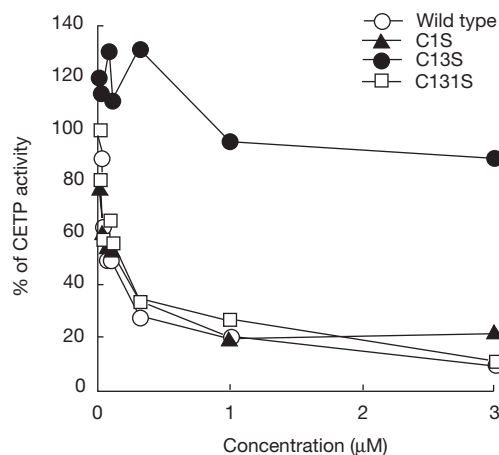


Figure 1 Effect of JTT-705 on the activity of wild-type and mutant CETP proteins. Recombinant human wild-type and mutant CETP proteins were prepared and purified from the serum-free conditioned medium of recombinant Raji cells (wild type) or PAI cells (mutants) expressing the relevant cDNA. The CETP activity in these proteins were determined as described in Methods.

Table 1 Effect of various CETP inhibitors on CETP activity in human plasma

Compound		Plasma CETP inhibition IC ₅₀ (μM)
1	R = -CH ₃	500
2	R = -CH(CH ₃) ₂	41
3	R = -C(CH ₃) ₃	13
4	R =	2
5		> 300
6		> 300
7	R' = -H	5
8	R' = -CH ₃	> 300
JTT-705	R' = -COCH(CH ₃) ₂	9

IC₅₀: concentration achieving 50% inhibition of CETP-mediated cholesteryl ester transfer from HDL to VLDL/LDL in human plasma.

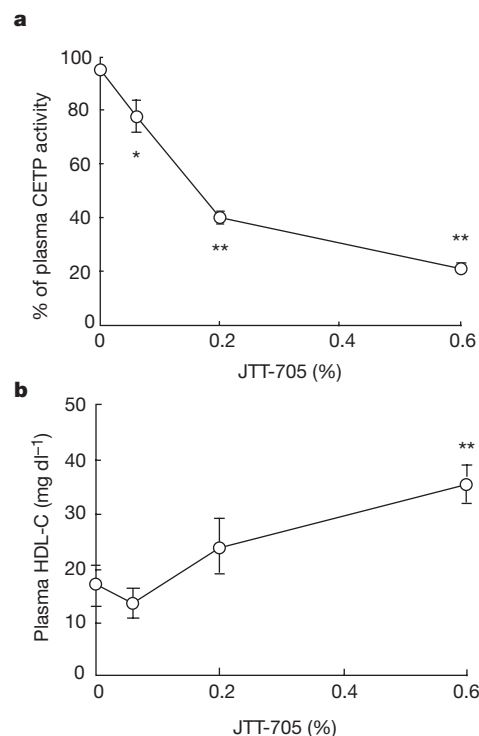


Figure 2 Effect of JTT-705 on plasma CETP activity and HDL-C level in rabbits fed regular chow. **a**, CETP activity. **b**, HDL-C level. JTT-705 (0.06%, 0.2% or 0.6%) was added to the diet for seven days. Plasma samples were taken before administration and 16 h after completing the whole administration period. The percent CETP activity was calculated as [(activity after administration)/(activity before administration) × 100]. Values represent the mean ± s.e.m. (n = 4). Asterisk, P < 0.05; double asterisk, P < 0.01 versus the control group (Dunnett's test).

the plasma of treated rabbits, an immunoassay using two monoclonal antibodies indicated that administration of JTT-705 caused modification of CETP, as we could detect CETP in the plasma of untreated rabbits, but could not identify it after treatment with JTT-705. This indicated that JTT-705 had induced some structural change in CETP, preventing binding with the antibody. After we confirmed the *in vivo* inhibitory effect of JTT-705 on CETP, we next examined its effect on plasma HDL cholesterol (HDL-C) and plasma CETP activity in Japanese white rabbits fed regular rabbit chow. After administration in the diet for 7 days, JTT-705 dose-dependently inhibited CETP activity and increased HDL-C (Fig. 2). As the per cent change in HDL-C in these rabbits was inversely correlated with CETP activity ($r = -0.695$, $P < 0.01$), whereas JTT-705 did not increase HDL-C in rats (which do not possess CETP), this compound apparently increased HDL-C through its specific inhibition of CETP.

Administration of 0.6% JTT-705 in the diet significantly increased plasma HDL-C, so we administered 0.75% JTT-705 (a mean dose of 255 mg kg^{-1}) to allow for an increase in body weight in the next long-term study. We used cholesterol-fed rabbits, because the development of atherosclerosis in this model has been well characterized¹⁸. A diet containing 0.2% cholesterol was given for five weeks (100 g per day) to establish hyperlipidaemia, followed by a 0.2% cholesterol diet containing one of the test compounds (0.75%

JTT-705 or 0.0075% simvastatin) for six months. The control compound, simvastatin, is a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor and a typical LDL-lowering drug.

The effects of the two compounds on plasma CETP activity, plasma lipid levels, serum HDL subfractions, the serum apolipoprotein A-I (apoA-I) level and toxicological parameters are summarized in Table 2. JTT-705 showed a continuous inhibitory effect on CETP activity during treatment. Although simvastatin also caused a decrease in CETP activity, the change was not significant. JTT-705 caused a 90% increase in HDL-C compared with the control value, and also decreased non-HDL-C by 40–50%. On the other hand, simvastatin markedly decreased non-HDL-C by 50–70%, but only slightly increased HDL-C by 28%. Lipoprotein analysis by ultracentrifugation revealed that JTT-705 significantly increased both HDL₂-C (170%) and HDL₃-C (59%), whereas simvastatin only increased HDL₃-C (38%). Serum apoA-I was significantly increased by both drugs (78% by JTT-705 and 41% by simvastatin). As a result, the atherogenic index (the ratio of non-HDL-C to HDL-C) was almost the same after six months of treatment with either drug. Figure 3 shows the lipoprotein profile determined from pooled plasma by fast performance liquid chromatography (FPLC) analysis after three months of treatment. JTT-705 increased the HDL-C level, particularly in large HDL particles,

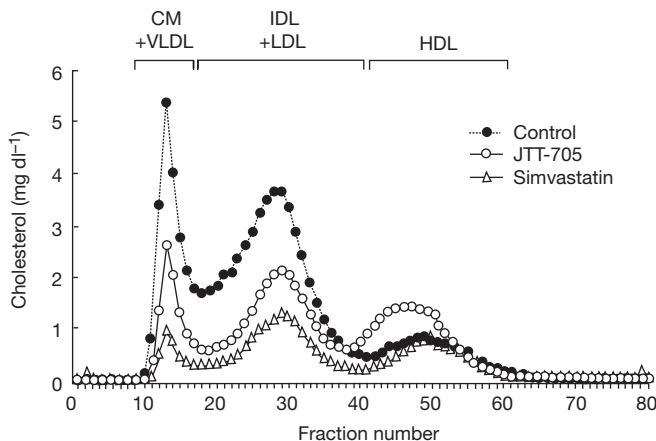


Figure 3 Cholesterol distribution in plasma lipoproteins from control, JTT-705-treated and simvastatin-treated rabbits at three months. The relative positions of chylomicron (CM) + VLDL, intermediate density lipoprotein (IDL) + LDL and HDL were determined after the fractions separated by ultracentrifugation were individually chromatographed on two Superose 6 columns, and are indicated at the top of the graph.

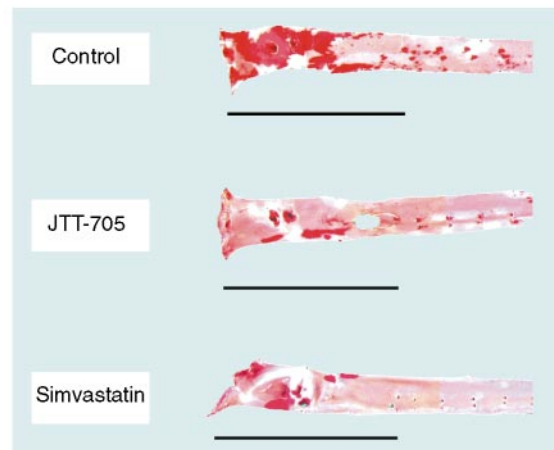


Figure 4 Typical photographs of aortas from control, JTT-705-treated and simvastatin-treated rabbits. The line below each aorta indicates the extent of the aortic arch (origin to the first set of intercostal arteries).

Table 2 Effects of JTT-705 and simvastatin on plasma CETP activity, plasma lipids, serum HDL subfractions, serum apoA-I and aortic atheroma in rabbits fed a 0.2% cholesterol diet

Group	N	Time (months)	Body weight (kg)	CETP activity* (% of initial activity)	Total cholesterol (mg dl ⁻¹)	Plasma† HDL-C (mg dl ⁻¹)	Atherogenic index	HDL ₂ -C (mg dl ⁻¹)	Serum‡ HDL ₃ -C (mg dl ⁻¹)	ApoA-I (mg dl ⁻¹)	Lesion area in the aortic arch§ (%)	Liver weight (g)
Control	10	0	2.40 ± 0.04	100 ± 0	274 ± 30	17.5 ± 1.7	16.6 ± 2.7					
		3	3.00 ± 0.06	77 ± 5	153 ± 25	17.0 ± 1.5	8.3 ± 1.4					
		6	3.24 ± 0.07	96 ± 8	129 ± 50	15.2 ± 1.8	7.3 ± 2.8	6.5 ± 0.9	8.1 ± 1.0	27.4 ± 3.1	30.3 ± 11.5	103 ± 8
JTT-705	10	0	2.41 ± 0.04	100 ± 0	263 ± 29	17.5 ± 1.3	14.4 ± 1.8					
		3	2.92 ± 0.07	31 ± 4¶	103 ± 14	33.0 ± 4.2¶	2.4 ± 0.6¶					
		6	3.23 ± 0.07	2.9 ± 7.7¶	98 ± 10	30.0 ± 3.6¶	2.6 ± 0.5	17.6 ± 2.9¶	12.9 ± 0.7¶	48.8 ± 3.2¶	9.2 ± 5.9#	97 ± 4
Simvastatin	10	0	2.37 ± 0.02	100 ± 0	269 ± 34	16.8 ± 1.6	16.1 ± 2.5					
		3	2.95 ± 0.03	94 ± 7	57 ± 10	18.1 ± 1.6	2.1 ± 0.3¶					
		6	3.26 ± 0.04	68 ± 13	77 ± 16	19.5 ± 2.8	2.7 ± 0.5	8.8 ± 1.8	11.2 ± 1.0	38.6 ± 2.0¶	5.9 ± 2.1#	88 ± 5

JTT-705 (0.75%) or simvastatin (0.0075%) was added to the diet for six months after preloading with a 0.2% cholesterol diet for five weeks.

* Plasma samples were taken before administration and 7 h after administration at both three and six months. CETP activity was determined as described in Methods and the percent CETP activity was calculated as [(activity at the time of assessment/initial activity) × 100].

† Plasma samples were taken before administration and 16 h after administration at three and six months. HDL-C and total cholesterol (TC) were measured using a commercial available kit (Boehringer Mannheim) and the atherogenic index was calculated as [(TC) - (HDL-C)]/(HDL - C).

‡ Serum samples were taken 16 h after administration at six months. The cholesterol content in the HDL₂ fraction ($1.063 \text{ g ml}^{-1} < d < 1.125 \text{ g ml}^{-1}$) and the HDL₃ fraction ($1.125 \text{ g ml}^{-1} < d < 1.21 \text{ g ml}^{-1}$) was measured using the above-mentioned kit. The apoA-I level was measured by a competitive immunoassay using goat anti-rabbit apoA-I antiserum.

§ At the end of the experimental period, the aortas were removed and stained with 0.5% sudan IV. The severity of aortic atherosclerosis was evaluated as the per cent lesion area relative to the intimal surface area.

Values represent the mean ± s.e.m. Significantly different from the control group by the Tukey-Kramer test (|| $P < 0.05$, ¶ $P < 0.01$) or by the Mann-Whitney U-test (# $P < 0.05$).

and decreased non-HDL-C (VLDL and LDL). Thus, we synthesized a potent CETP inhibitor and showed that this inhibitor could increase HDL-C and decrease non-HDL-C *in vivo*. As far as we know, this is the most effective HDL-C-increasing agent reported so far, except for an anti-CETP monoclonal antibody¹⁹.

In our study, inhibition of CETP by JTT-705 led to elevation of HDL-C and reduction of non-HDL-C (or LDL-C), as also occurs in human CETP deficiency (intron 14 homozygotes)²⁰ and in rabbits treated with a CETP antisense oligonucleotide⁸. However, the extent of the changes to HDL-C and non HDL-C differed between JTT-705-treated rabbits, genetic CETP deficiency²⁰ and antisense oligonucleotide-treated rabbits⁸ because of the different mechanisms causing the reduction in CETP activity. CETP is absent in genetic CETP deficiency, whereas CETP production is suppressed by the antisense oligonucleotide and JTT-705 inhibits intrinsic plasma CETP. In a clinical study, JTT-705 (600–900 mg per day for 14 days) caused a 40–45% increase in HDL-C and a 15–20% decrease in LDL-C. Therefore, our findings in rabbits may reasonably be extrapolated to predict the changes in plasma lipids in humans treated with this drug.

Table 2 and Fig. 4 show the effect of JTT-705 on the progression of atherosclerosis. After six months of treatment, we measured the area of atherosclerotic lesions in the aortic arch. JTT-705 caused a 70% decrease in lesion area, and its effect was similar to that of simvastatin, which caused an 80% decrease in lesion area. There have been many reports of the anti-atherogenic effects of HDL-C^{21–24}. Although JTT-705 caused a smaller reduction in non-HDL-C than that achieved by simvastatin, it produced a more marked elevation of HDL-C. In addition, our *in vitro* studies showed that JTT-705 (20 μ M) did not influence the activities of enzymes related to lipid metabolism, such as HMG-CoA reductase and acyl-CoA:cholesterol acyltransferase. Furthermore, the drug showed no significant *in vivo* toxicity in rabbits, on the basis of assessment of body weight, liver weight (Table 2), liver function (ALT and AST) and renal function (creatinine and urea nitrogen). Both the elevation of HDL-C and the reduction of non-HDL-C may contribute to the anti-atherogenic effect of JTT-705. These results also indicate that CETP may be important in determining the composition of plasma HDL-C and non-HDL-C (or LDL-C), that is, the atherogenic index, and in the formation of aortic atheroma in rabbits.

In conclusion, inhibition of endogenous CETP activity was able to inhibit the progression of atherosclerosis in rabbits, a finding that confirms the atherogenicity of CETP^{4,9}. JTT-705 is a potent CETP inhibitor, which has the potential to be used as an anti-atherogenic agent. □

Methods

Recombinant human wild-type and mutant CETP

Mutant proteins, in which the cysteine at residue 1 (C1S), 13 (C13S) or 131 (C131S) was substituted with serine, were generated as described²⁵. Recombinant human wild-type and mutant CETP proteins were prepared from the serum-free conditioned medium of recombinant Raji cells (wild type) or PAI cells (mutants) expressing the relevant complementary DNA. Proteins were purified from the medium by hydrophobic column chromatography, gel-filtration column chromatography and immunoaffinity column chromatography using an anti-human CETP monoclonal antibody (JHC1, prepared by JT Inc.). Purified proteins exhibited a single band on SDS–polyacrylamide gel electrophoresis. The concentration of wild-type protein was determined by amino-acid analysis, and the concentrations of the mutant proteins were assessed from the absorbance measured at 280 nm on a spectrophotometer using wild-type protein as the standard. The activity of the wild-type and CETP mutant proteins was determined as described²⁶. In brief, each protein (10 ng) was incubated for 15 h at 37 °C in the presence of [³H]cholesteryl ester-labelled HDL (0.18 μ g cholesterol), VLDL/LDL (18 μ g cholesterol) and a test compound (dissolved in dimethyl sulphoxide). The volume was adjusted to 600 μ l with Tris-buffered saline (pH 7.4). After precipitation of apolipoprotein B (apoB)-containing lipoproteins by dextran sulphate (0.038%) and MgCl₂ (19 mM), half of the supernatant was removed and its radioactivity was counted in a liquid scintillation counter. CETP activity was determined from the decrease in radioactivity versus that of the blank containing no CETP.

Human plasma CETP assay

Human plasma (100 μ l) was incubated at 37 °C or 4 °C for 4 h in the presence of

[³H]cholesteryl ester-labelled HDL and one of the test compounds (dissolved in a mixture of N-methyl-2-pyrrolidinone and polyethylene glycol 400 (1:1)). After precipitation of apoB-containing lipoproteins by dextran sulphate (0.075%) and MgCl₂ (37.5 mM), half of the supernatant was removed and its radioactivity was counted in a liquid scintillation counter. CETP activity was determined from the decrease in radioactivity at 37 °C versus that at 4 °C.

Ex vivo and in vivo study

This experiment complied with the guidelines on animal experimentation of our laboratories. Eight-week-old male Japanese white rabbits were used. JTT-705 and simvastatin (Banyu Pharmaceuticals) were added to regular rabbit chow or to a 0.2% cholesterol diet. Plasma samples were taken at the designated times, and CETP activity was determined as described²⁶. In brief, an aliquot of plasma was incubated with [³H]cholesteryl ester-labelled HDL (0.127–0.266 μ g cholesterol) and VLDL/LDL (12.7–16.6 μ g cholesterol) at 37 °C for 15 h. Then the volume was adjusted to 600 μ l with Tris-buffered saline (pH 7.4). After precipitation of apoB-containing lipoproteins using dextran sulphate (0.027%) and MgCl₂ (27 mM), half of the supernatant was removed and the radioactivity was counted in a liquid scintillation counter. CETP activity was determined from the decrease in radioactivity versus that of the blank containing no plasma. Plasma HDL was separated from apoB-containing lipoproteins by precipitation with 13% polyethylene glycol 6000²⁷. HDL-C and total cholesterol (TC) were measured using a commercial kit (Boehringer Mannheim). The atherogenic index was calculated as [(TC) – (HDL – C)]/(HDL – C). Serum HDL subfractions were determined after six months by serial ultracentrifugation. The cholesterol content of the HDL₂ (1.063 g ml⁻¹ < d < 1.125 g ml⁻¹) and HDL₃ (1.125 g ml⁻¹ < d < 1.21 g ml⁻¹) fractions was measured using the above-mentioned kit. The serum apoA-I level at six months was measured by a competitive immunoassay using goat anti-rabbit apoA-I antiserum.

Lipoprotein analysis

An aliquot (200 μ l) of pooled plasma obtained from control, JTT-705-treated and simvastatin-treated rabbits at three months was added to an FPLC system consisting of two Superose 6 columns (Pharmacia) in series²⁸. Plasma lipoproteins were eluted with 50 mM phosphate-buffered saline containing 1 mM EDTA and 0.02% sodium azide, and were fractionated into 0.375-ml aliquots. The cholesterol content in each fraction was measured using a commercial kit (Boehringer Mannheim).

Histological examination

At the end of the experimental period, the aortas of the rabbits were removed and stained with 0.5% sudan IV. Quantification of the lesion area in the aortic arch (origin to the first set of intercostal arteries) was performed with a colour image analyser and the severity of aortic atherosclerosis was evaluated as the percent lesion area relative to the intimal surface area.

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PML regulates p53 acetylation and premature senescence induced by oncogenic Ras

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The tumour suppressor p53 induces cellular senescence in response to oncogenic signals¹. p53 activity is modulated by protein stability and post-translational modification, including phosphorylation and acetylation². The mechanism of p53 activation by oncogenes remains largely unknown. Here we report that the tumour suppressor PML regulates the p53 response to oncogenic signals. We found that oncogenic Ras upregulates PML expression, and overexpression of PML induces senescence in a p53-dependent manner. p53 is acetylated at lysine 382 upon Ras expression, an event that is essential for its biological function. Ras induces re-localization of p53 and the CBP acetyltransferase within the PML nuclear bodies and induces the formation of a trimeric p53–PML–CBP complex. Lastly, Ras-induced p53 acetylation, p53–CBP complex stabilization and senescence are

lost in PML^{−/−} fibroblasts. Our data establish a link between PML and p53 and indicate that integrity of the PML bodies is required for p53 acetylation and senescence upon oncogene expression.

PML is involved in the pathogenesis of acute promyelocytic leukaemia^{3–7}. Mice with a targeted disruption of the PML locus (PML^{−/−}) display increased incidence of carcinomas after treatment with tumour-promoting agents⁸. We investigated whether PML functions in the cellular fail-safe mechanisms of tumour suppression by expressing an activated Ras oncogene (RasV12) in PML^{−/−} primary mouse embryo fibroblasts (MEF). Prolonged expression of RasV12 in MEFs induces a permanent cell-cycle arrest, indistinguishable from cellular senescence⁹. We infected PML^{−/−} and wild-type MEFs with a pBABE-PURO retrovirus containing RasV12, cultured them for 4 days under pharmacological selection, and counted cells for the subsequent 12 days. Wild-type MEFs expressing oncogenic Ras ceased to proliferate at subconfluent densities (Fig. 1Aa)¹. Cells became flat and enlarged, did not incorporate labelled nucleotides (bromodeoxyuridine; BrdU) (Fig. 1B), and became positive for acidic β -galactosidase (SA- β -gal), a marker of cellular senescence⁹ (Fig. 1C). Conversely, Ras only slightly inhibited growth of PML^{−/−} MEFs and did not alter BrdU incorporation (Fig. 1Ab and 1B). The frequency of SA- β -gal⁺ cells was markedly lower in Ras-expressing PML^{−/−} MEFs than in controls (Fig. 1C). Therefore, PML expression is required for Ras-induced senescence.

Overexpression of PML (or green fluorescent protein (GFP)-tagged PML) in human or murine fibroblasts arrested proliferation (Fig. 1D). PML-overexpressing fibroblasts remained viable for several weeks, did not incorporate BrdU, accumulated in G1, and did not undergo apoptosis (data not shown). Morphologically, they became flat and enlarged and were strongly positive for SA- β -gal (Fig. 1E). The extent of growth suppression (Fig. 1Aa) and induction of SA- β -gal positivity (Fig. 1C) by PML or Ras were comparable.

PML localizes to specific nuclear subdomains, the so-called nuclear bodies (12.1 $\pm$ 3.6 bodies per nucleus of 229 $\pm$ 50.9 nm diameter, Fig. 1F)¹⁰. RasV12 expression increased the number (30 $\pm$ 10) and size (601.7 $\pm$ 124.4 nm) of the PML nuclear bodies, with the appearance of annular structures (1–2 in 60–70% of infected cells; 1–2 μ m diameter), similar to those observed upon PML overexpression (2–10 structures per cell, diameter 1,302 $\pm$ 147 nm; Fig. 1F). These changes were accompanied by a progressive increase in PML protein levels (Fig. 1G). It therefore appears that PML is part of the signalling pathway of oncogene-induced senescence.

p53 and p16 are vital components of the intracellular senescence pathways^{1,11–13}. PML overexpression induced upregulation of p53 (6–8-fold) and of p21CIP1 (2–3-fold), a transcriptional target of p53 (ref. 14). Although PML and Ras induced equivalent levels of p53 and p21CIP1, PML did not influence p16 expression (Fig. 2A). PML did not induce growth arrest or senescence in p53^{−/−} or p21^{−/−} fibroblasts (Fig. 2B), although the typical nuclear bodies staining pattern of native PML was discernable (Fig. 2C). Therefore, p53 and p21 are crucial for PML-induced senescence.

Ras induced comparable amounts of p19ARF and p53 proteins in wild-type and PML^{−/−} MEFs (Fig. 2D)¹⁵; however, a slight reduction of p21 upregulation occurred in PML^{−/−} MEFs (9.6-fold and 5.4-fold increase in wild-type and PML^{−/−} MEFs, respectively) (Fig. 2D), suggesting that PML regulates p53 activity rather than stability. p53 activity is regulated by phosphorylation and acetylation^{2,16}. Two sites of acetylation, K320 and K382 (corresponding to mouse K317 and K379) have been identified^{16,17}. *In vivo*, PML physically associates with CBP/p300, acetyltransferases that modify p53 at K382 (refs 16–18; and unpublished observations). We investigated whether p53 acetylation is relevant for its activation by Ras or PML. Transient transfection of wild-type p53 in p53^{−/−} MEFs yielded dose-dependent transcriptional activation (from 8- to 90-fold) of a synthetic p53-target promoter (Fig. 3A). p53 mutants

defective for acetylation at K382 (p53^{K382R}) or K320 (p53^{K320R}) yielded similar levels of promoter activation¹⁷. Co-expression of Ras or PML increased the transcriptional activation of wild-type p53, but not that of p53^{K382R} or p53^{K320R} (Fig. 3A), indicating that acetylation may be required for the optimal activation of p53 by Ras or PML.

We then evaluated the status of p53 acetylation during spontaneous and Ras- or PML-induced senescence. The levels of immunoprecipitated p53 were normalized by western blotting and acetylation assessed using antibodies specific for acetylated K320 or K382 (ref. 16). In serially passaged wild-type MEFs, a progressive increase in acetylation at both K320 and K382 occurred (Fig. 3B). Expression of oncogenic Ras or PML induced a modest, yet reproducible, increase in acetylation of p53 at K382 (Fig. 3C), but not K320 (data not shown).

To investigate the biological relevance of p53 acetylation at K382, p53^{-/-} MEFs were infected with Ras and then re-infected at high efficiency (>90%) with either empty, p53- or p53^{K382R}-expressing PINCO retroviruses. PINCO expresses GFP as a marker of infection¹⁹. Ras-PINCO double-infected cells cloned at high efficiency in methylcellulose, and greater than 95% of the colonies were GFP positive. p53 suppressed Ras-induced transformation, as Ras-p53 double-infected cells produced less than 10% of the colonies seen with the control, of which all were GFP negative. We observed a significant increase in the number of colonies derived from Ras-p53^{K382R}-infected cells (25–30% of Ras-PINCO control; 50% GFP⁺) (Fig. 3E), suggesting that acetylation at K382 contributes significantly to the ability of p53 to suppress Ras-mediated transformation.

A striking reduction in Ras-induced p53 acetylation at K382 was observed in PML^{-/-} MEFs (Fig. 3C). In agreement, Ras-induced p53 transactivation was also significantly reduced in PML^{-/-} cells (Fig. 3D). Therefore, PML expression is required for both K382

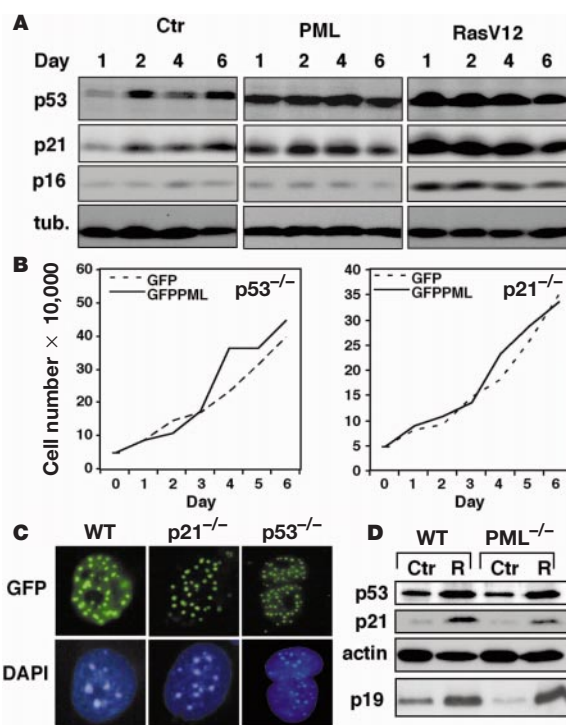


Figure 2 PML-induced senescence is dependent on p53. **A**, Western blot analysis of p53, p21, p16 and tubulin of WI38 infected with vector (Ctr), PML or RasV12. **B**, Growth curve of p53^{-/-} or p21^{-/-} fibroblasts infected with GFP or GFP-PML. **C**, Indirect immunofluorescence of wild-type (WT), p21^{-/-} and p53^{-/-} fibroblasts 10 days after infection with GFP-PML. Lower panels show DAPI staining. **D**, Western blot analysis of p21, p53, actin and p19ARF upon infection of wild-type and PML^{-/-} fibroblasts with vector (Ctr) or RasV12 (R).

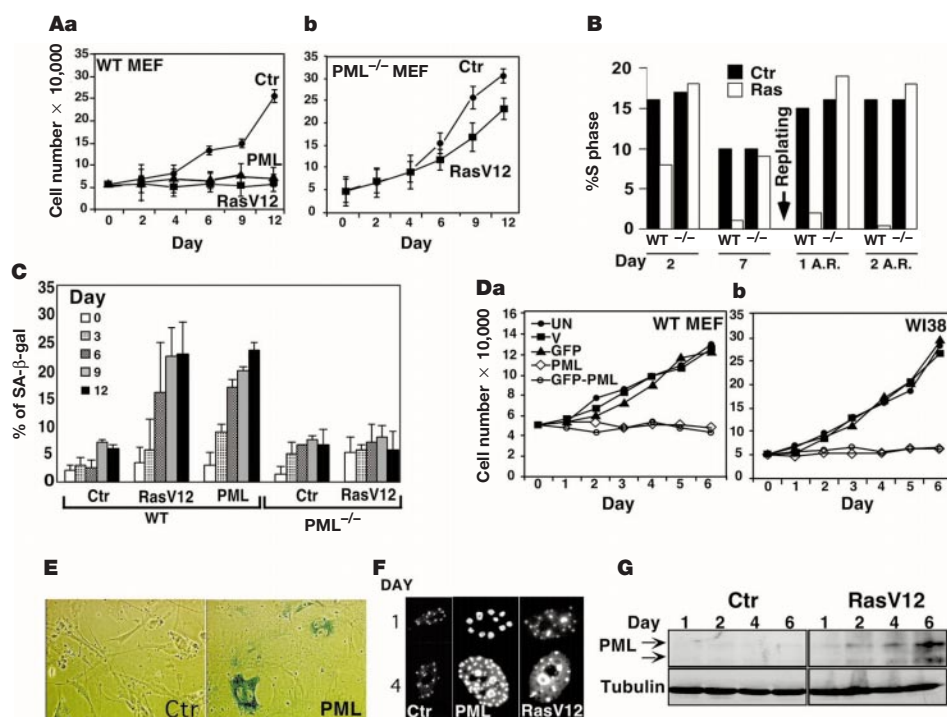


Figure 1 PML is required for Ras-induced senescence in primary cells. **A**, Growth curve of wild-type (WT; **a**) or PML^{-/-} (**b**) fibroblasts infected with vector (Ctr), PML or RasV12 (infection efficiencies higher than 60%). Day 0 is the first day after selection. A representative of four experiments giving comparable results is shown. **B**, BrdU incorporation of wild-type and PML^{-/-} MEFs infected with vector (Ctr) or RasV12. Cells were replated after confluency; A.R., days after replating. **C**, Histogram comparing

RasV12- and PML-induced senescence, as assayed by β-gal activity. **D**, Growth curves of MEFs (**a**) and WI38 (**b**) fibroblasts, uninfected (UN), infected with empty vector (V), or GFP, PML or GFP-PML. **E**, β-gal staining of MEFs infected with vector (Ctr) or PML. **F**, Indirect immunofluorescence of PML in WI38 cells infected with vector (Ctr), PML or RasV12, 1 or 4 days after selection. **G**, Western blot analysis of endogenous PML (arrow) in WI38 after infection with vector (Ctr) or RasV12.

acetylation and optimal p53 transactivation by oncogenic Ras.

As p53 can form stable complexes with CBP/p300 *in vivo*^{16,17,20,21}, we investigated whether PML or Ras regulates the stoichiometry of the p53–CBP complex. Increasing amounts of the p53–CBP complex were recovered upon infection with PML or Ras in wild-type cells. Ras-induced upregulation of the p53–CBP complex was markedly reduced in PML^{-/-} fibroblasts (Fig. 4A). Furthermore, the amount of recovered PML–CBP complex increased markedly after PML or Ras expression (Fig. 4B), suggesting that a p53–PML–CBP tricomplex may occur *in vivo*. To prove the existence of the tricomplex, we carried out sequential immunoprecipitation studies using anti-CBP and anti-p53 antibodies. Anti-PML immunoreactive polypeptides were detected in the sequential anti-CBP/anti-p53, but not in the control anti-CBP/anti-haemagglutinin A (HA), immunoprecipitates (Fig. 4C). Increased amounts of CBP–p53–PML complex were recovered from cells infected with RasV12 (Fig. 4C), indicating that Ras favours the formation of a p53–PML–CBP/p300 complex *in vivo*.

Finally, we analysed the effects of PML or Ras on the nuclear localization of p53 and CBP (Fig. 5). Vector-infected cells displayed nuclear diffuse staining patterns of p53 and CBP (Fig. 5Aa, m) that did not colocalize with PML (Fig. 5Aa, l). Large aggregates of p53 occurred upon expression of Ras or PML (2.7 ± 1.9 aggregates, diameter of 740 ± 129 nm in 20% of Ras-infected cells; 4.5 ± 1.98 aggregates, diameter of $1,058.1 \pm 361.5$ nm in all PML-overexpressing cells) (Fig. 5Ae, h) that colocalized with PML (Fig. 5d, g). CBP formed aggregates upon Ras (25.7 ± 7.4 aggregates per cell; diameter 593.3 ± 95.7 nm) (Fig. 5Ap) and PML (20.6 ± 5.2 aggregates per cell; diameter $1,151 \pm 291$ nm) (Fig. 5As) expression, which colocalized with PML^{18,22} (Fig. 5Ao, r). Triple staining confocal analysis demonstrated colocalization of PML, p53 and CBP (Fig. 5B). These colocalization foci correspond to the PML nuclear bodies, as demonstrated by the presence of Daxx, another PML nuclear body component (ref. 23; and data not shown). In conclusion, expression of PML or Ras induces colocalization of PML, p53 and CBP within a subset of the PML nuclear bodies. Ras failed to induce CBP aggregates in PML^{-/-} MEFs, in which

the nuclear bodies are disrupted^{23,24} (Fig. 5C). All together these findings suggest that intact PML nuclear bodies are required for oncogene-induced reorganization of CBP and p53, stable p53–PML–CBP/p300 complex formation, p53 acetylation and induction of replicative senescence.

PML failed to modify p53 acetylation levels in *in vitro* assays (data not shown), indicating that other factors may be required for an active p53–PML–CBP/p300 complex *in vivo*. Of note, PML induces caspase-independent apoptosis in immortalized/transformed

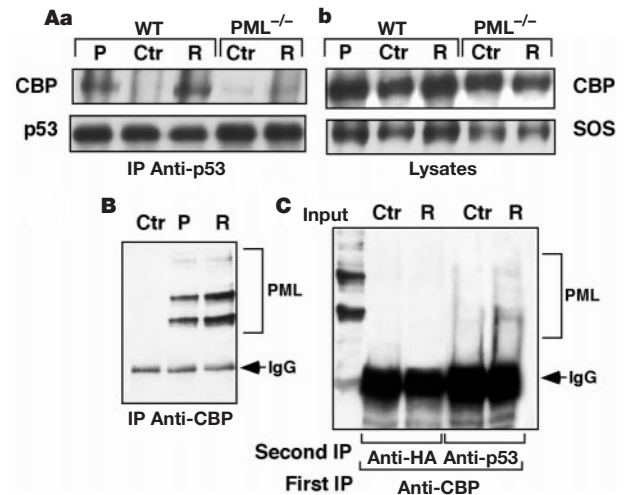


Figure 4 RasV12 induces complex formation between p53, PML and CBP. **Aa**, Anti-CBP western blots of anti-p53 immunoprecipitates from vector (Ctr), PML (P) or RasV12 (R)-infected wild-type or PML^{-/-} MEFs (upper panel); normalization of p53 (lower panel); **Ab**, anti-CBP or anti-SOS western blots of total lysates. **B**, Anti-PML western blots of anti-CBP immunoprecipitates from WI38 fibroblasts infected with vector (Ctr), PML (P) or RasV12 (R). **C**, Anti-PML western blots of sequential anti-CBP/anti-p53 (or anti-HA) immunoprecipitates. Cells were crosslinked with 0.5 mM DTBP (Dimethyl 3,3'-dithiobispropionamide-2-HCl; Pierce).

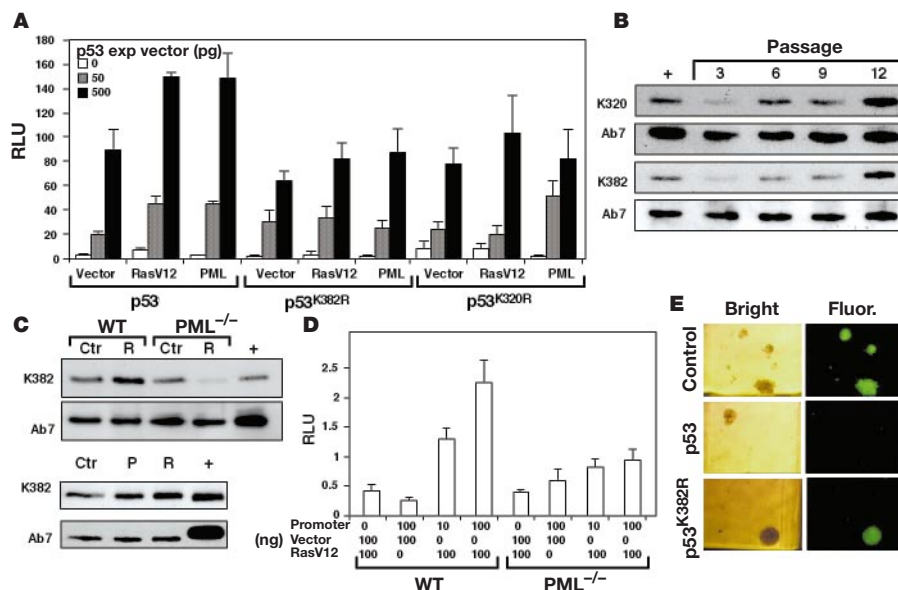


Figure 3 p53 acetylation accompanies senescence and is downregulated in PML^{-/-} fibroblasts. **A**, p53 transcriptional activity in p53^{-/-} fibroblasts transfected with the indicated amounts of wild-type p53, p53^{K382R} or p53^{K320R} constructs in the presence of 100 ng of control, or RasV12- or PML-expressing vectors. Values represent means of triplicate experiments normalized by co-transfected β -gal. **B**, Western blots of p53 acetylation at K320 or K382, from lysates of serially passaged fibroblasts. Positive

controls are derived from ultraviolet-irradiated A549 cells. **C**, Western blot analysis of levels of acetylation at K382 from wild-type and PML^{-/-} fibroblasts infected with vector (Ctr) or Ras (R). **D**, p53 transcriptional activity in wild-type and PML^{-/-} fibroblasts transfected with vector or RasV12. **E**, Bright-field or fluorescence analysis of methylcellulose colonies of p53^{-/-} fibroblasts double-infected with RasV12–PINCO (Ctr), RasV12–p53 wild type or RasV12–p53^{K382R}.

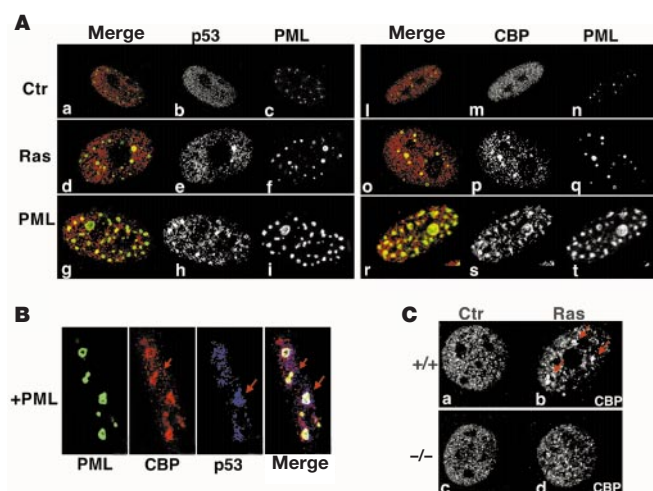


Figure 5 Colocalization of PML, CBP and p53 after expression of PML or RasV12. Indirect immunofluorescence of WI-38 (**A, B**) or MEF (**C**) is shown of confocal images of single sections (0.3 μ m). **A**, Immunofluorescence of WI-38 infected with empty vector (Ctr; **a–c, i–n**), RasV12 (Ras; **d–f, o–q**) or PML (**g–i, r–t**). PML was visualized with anti-Myl (**c, f, i**) or PGM-3 (**n, q, t**) antibodies. Green fluorescence, PML; red fluorescence, p53 or CBP. Colocalization is indicated in yellow. **B**, Triple immunofluorescence of PML, CBP and p53 in WI-38 infected with GFP-PML. Green fluorescence, GFP; red fluorescence, A22; blue fluorescence, DO-1. Colocalization is shown in Merge. Green and red colocalization is yellow; green and blue is aquamarine; red and blue is violet; triple staining is white. The narrow arrow indicates an area of PML and CBP staining, the wide arrow triple staining. **C**, CBP staining of wild-type (**a, b**) and PML^{−/−} (**c, d**) fibroblasts infected with empty vector (Ctr) or RasV12.

cells²⁵, in which the ARF/p53 pathway is disrupted²⁶, suggesting that the biological outcome of PML activation, that is, senescence or apoptosis, depends on the integrity of these pathways. Investigating how the PML-nuclear-body structure alters upon activation of other p53 functions, such as the control of genomic stability^{27–29}, will provide insight into nuclear functions and, in the specific context shown here, p53 regulation. □

Methods

Cells and antibodies

Infection of MEFs was carried out as described¹. Antibodies were anti-p21 (F-5; Santa Cruz), anti-p53 (DO-1, Santa Cruz; Ab-7, Calbiochem), anti-p53AC (421), anti-PML (PGM-3, Santa Cruz; anti-Myl³⁰), anti-tubulin (Santa Cruz), anti-actin (Signal Transduction Labs), anti-CBP (A22, Santa Cruz), anti-SOS (Santa Cruz) and anti p19ARF (ABCAM).

Transactivation

p53 activity was assayed using the pGL13LUC plasmid, and normalized using a co-transfected β -galactosidase construct.

Immunofluorescence

Antibodies were visualized with anti-mouse Alexa488 or anti-rabbit Alexa568 (Molecular Probes). In triple staining anti-mouse Cy5 (Jackson) was used. Images were acquired using a confocal 1024MRC BIORAD microscope equipped with a krypton–argon LASER.

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Functional link of BRCA1 and ataxia telangiectasia gene product in DNA damage response

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BRCA1 encodes a familial breast cancer suppressor that has a critical role in cellular responses to DNA damage^{1,2}. Mouse cells deficient for *Brca1* show genetic instability, defective G2–M checkpoint control and reduced homologous recombination^{3,4}. **BRCA1** also directly interacts with proteins of the DNA repair

machinery⁵ and regulates expression of both the *p21* and *GADD45* genes^{6–8}. However, it remains unclear how DNA damage signals are transmitted to modulate the repair function of BRCA1. Here we show that the BRCA1-associated protein CtIP^{9–12} becomes hyperphosphorylated and dissociated from BRCA1 upon ionizing radiation. This phosphorylation event requires the protein kinase (ATM) that is mutated in the disease ataxia telangiectasia¹³. ATM phosphorylates CtIP at serine residues 664 and 745, and mutation of these sites to alanine abrogates the dissociation of BRCA1 from CtIP, resulting in persistent repression of BRCA1-dependent induction of *GADD45* upon ionizing radiation. We conclude that ATM, by phosphorylating CtIP upon ionizing radiation, may modulate BRCA1-mediated regulation of the DNA damage-response *GADD45* gene, thus providing a potential link between ATM deficiency and breast cancer.

BRCA1 interacts with the transcriptional co-repressor complex of CtIP and CtBP through its BRCT domains, and DNA damage-induced dissociation of BRCA1 from this complex may be important for BRCA1 function⁹. To explore the mechanism regulating this interaction, we examined the phosphorylation of CtIP in T24 cells treated with ionizing radiation (IR), ultraviolet or methylmethane sulphonate (MMS). CtIP immunoprecipitated from extracts of treated cells showed slower migrating forms (Fig. 1a, compare lanes 3–5 with lane 2), which were especially prominent in cells γ -irradiated at the dose of 20–40 Gy (Fig. 1a, lane 3; and Fig. 1b). The alteration in electrophoretic mobility was attributed to phosphorylation, as it was sensitive to λ -phosphatase but inhibited by NaF and Na₃VO₄ (Fig. 1c). λ -Phosphatase-treated CtIP migrated faster than the CtIP immunoprecipitated from control cells (Fig. 1c, compare lanes 4 and 2), suggesting that CtIP is a phosphoprotein

that becomes hyperphosphorylated after exposure of cells to DNA-damaging agents.

The phosphatidylinositol3-OH kinase [PI(3)K] related family of serine/threonine protein kinases, which includes ATM and DNA-PK (DNA-dependent protein kinase), have been implicated in cell signalling events in response to DNA double-strand breaks^{13,14}. It appears that a PI(3) related kinase is involved in the phosphorylation of CtIP, as the IR-induced hyperphosphorylation of CtIP was inhibited in cells treated with the PI(3)K inhibitor, wortmannin (20 μ M) (Fig. 1d, lane 4). In two isogenic cell lines, M059J (with deficient DNA-PK activity) and M059K (with normal levels of DNA-PK activity)¹⁵, the IR-induced hyperphosphorylation of CtIP remains unchanged (data not shown); however, the hyperphosphorylated forms of CtIP appeared only in ATM wild-type GM00637G cells, but not in ATM-deficient GM09607A cells after IR (Fig. 1e). Two other ATM-deficient cell lines, GM05849B and GM02052C, showed identical results (Fig. 1f). Similarly, in two isogenic stable clones derived from ATM-deficient fibroblasts (AT221JE-T) carrying either vector alone (plasmid pEBS7) or Flag-tagged wild-type ATM (plasmid pEBS7-YZ5)¹⁶, CtIP hyperphosphorylation was restored only in cells expressing wild-type ATM (Fig. 1g, bottom panel, compare lanes 2 and 4). The presence of wild-type ATM in these cells was confirmed by western blot analysis (Fig. 1g, top panel). These observations suggest that ATM is responsible for the phosphorylation of CtIP following IR.

ATM has been shown to phosphorylate Ser 15 in the highly conserved amino terminus of p53 with the sequence¹⁰ VEPPLSQE₁₇ in response to IR^{17,18}. The amino-acid sequence of CtIP has six SQ sites bearing similar sequences surrounding Ser 15 of p53. Two of these serines, 664 and 745, are conserved between human and

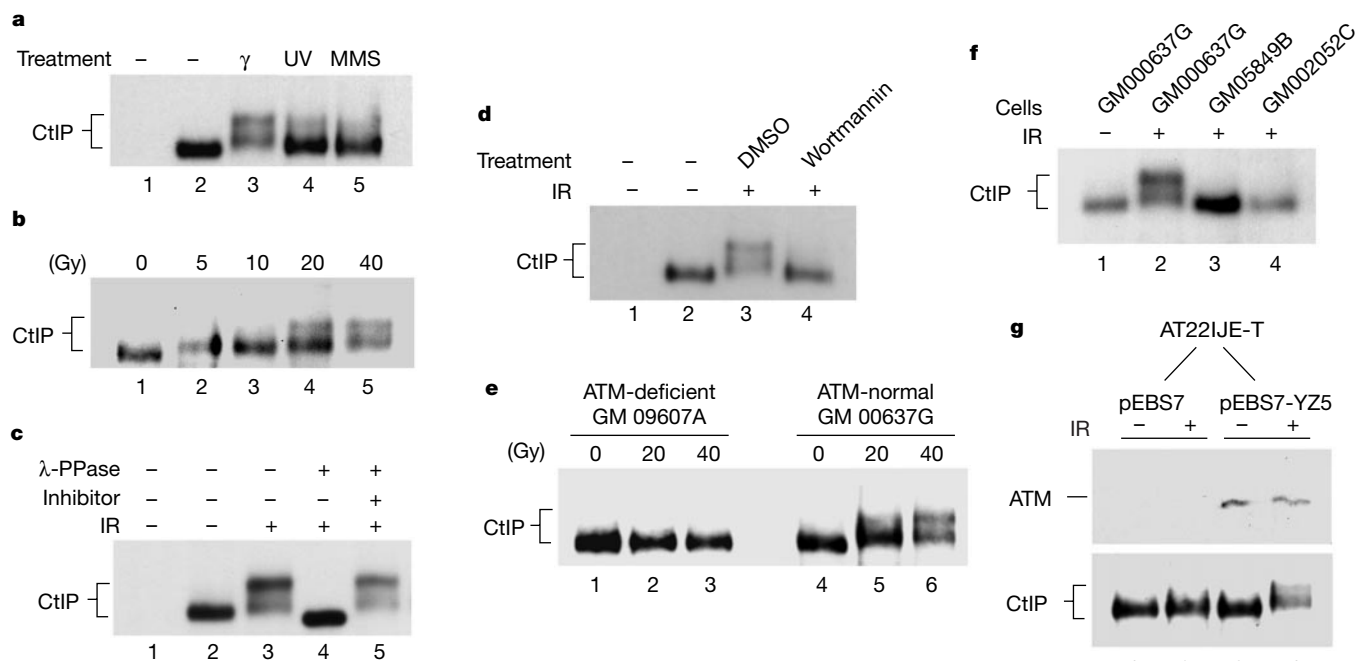


Figure 1 IR-induced phosphorylation of CtIP is ATM dependent. **a**, Change in CtIP gel mobility upon DNA damage. T24 cells were untreated (–) or treated with IR (γ), ultraviolet (UV) or MMS; extracts were immunoprecipitated using anti-CtIP antibody (lanes 2–5) and immunoblotted for CtIP. **b**, Dose-dependent IR phosphorylation of CtIP. T24 cells treated with IR (5–40 Gy) were immunoblotted for CtIP. **c**, Changes in mobility are due to the phosphorylation of CtIP. CtIP immune complexes from untreated (–) or γ -irradiated (+) T24 cells were untreated or treated with λ -phosphatase $\pm$ phosphatase inhibitors, as indicated (lanes 2–5), and immunoblotted with anti-CtIP antibodies. **d**, Inhibition of CtIP phosphorylation by wortmannin. T24 cells were untreated (lanes 1 and 2), or pre-incubated with either dimethyl-sulphoxide (DMSO; lane 3) or 20 μ M wortmannin (lane 4) before IR (40 Gy); the extracts were then immunoprecipitated and blotted for CtIP (lanes

2–4). Extracts were immunoprecipitated using pre-immune serum in lane 1 (**a,c,d**). **e**, Phosphorylation of CtIP upon IR is compromised in ATM-deficient cells. Cell extracts from ATM-deficient (GM09607A, lanes 1–3) or normal human fibroblasts (GM00637G, lanes 4–6) treated with IR for the indicated dosages were immunoblotted for CtIP. **f**, Absence of CtIP hyperphosphorylation in additional ATM-deficient cell lines (GM05849B, lane 3; GM02052C, lane 4) upon IR (40 Gy). **g**, Ectopic expression of wild-type ATM in A-T cells restored the phosphorylation of CtIP upon IR. ATM-deficient AT221JE-T cells were stably transfected with pEBS7 (vector) or pEBS7-YZ5 (carrying Flag-tagged wild-type ATM cDNA). Extracts from IR-treated cells (40 Gy) were immunoblotted with monoclonal antibodies: 2C1, for ATM (top); C11 for CtIP (bottom).

mouse (Fig. 2a), and we mutated these to alanines. Recombinant glutathione S-transferase (GST)–CtIP proteins carrying either the double mutations [GST–CtIP(C-S664/745A)] or no mutation [GST–CtIP(C)] were expressed and isolated for *in vitro* ATM kinase assays^{17,18}. Immunoprecipitated Flag-tagged wild-type ATM was capable of phosphorylating recombinant GST–CtIP(C), but not GST–CtIP(C-S664/745A) (Fig. 2b, bottom panel, compare lanes 2 and 5), whereas inactive mutant ATM(D2870A) did not phosphorylate either GST–CtIP(C) or GST–CtIP(C-S664/745A)

(Fig. 2b, bottom panel, lanes 3 and 6). The presence of Flag-tagged ATM and ATM(D2870A) was confirmed by immunoblot analysis (Fig. 2b, top panel). These data indicate that ATM may directly phosphorylate CtIP on Ser 664 and Ser 745 *in vitro*.

To determine whether these residues on CtIP are phosphorylated *in vivo*, we transfected Flag-tagged CtIP(WT), CtIP(S664A) or CtIP(S664/745A) into human osteosarcoma U2OS cells and metabolically labelled these cells with inorganic ³²P-phosphate. After IR, CtIP(WT), CtIP(S664A) or CtIP(S664/745A) were immunopreci-

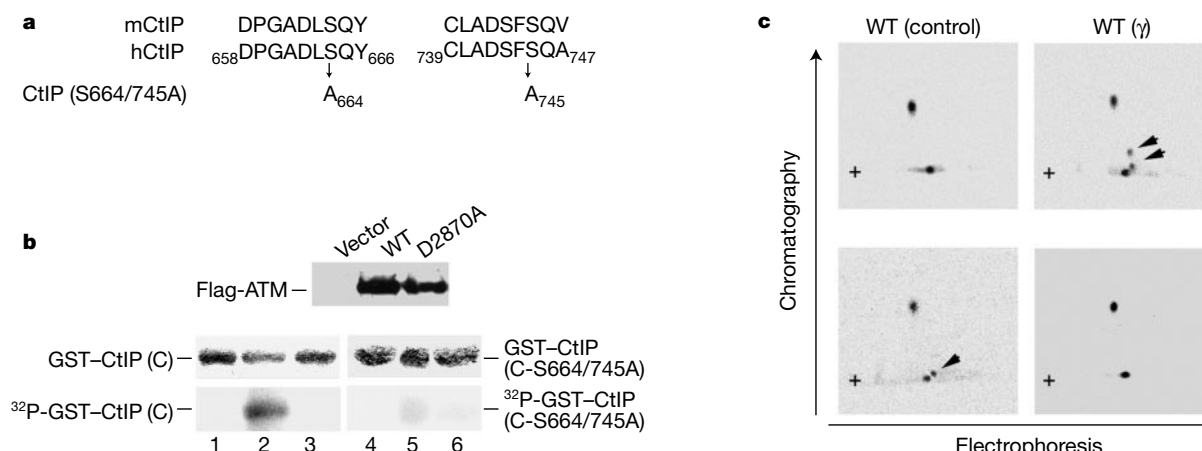


Figure 2 Identification of ATM phosphorylation sites on CtIP. **a**, Sequences of two potential ATM-phosphorylation sites in CtIP and the alignment between human (hCtIP) and mouse (mCtIP) CtIP. For the CtIP phosphorylation site mutant, CtIP(S664/745A), Ser 664 and Ser 745 were mutated to alanine. **b**, Phosphorylation of CtIP by ATM-associated kinase *in vitro*. 293 cells were transiently transfected with vector alone (lanes 1 and 4), or vectors expressing Flag-tagged wild-type ATM (WT) (lanes 2 and 5) or mutated ATM(D2870A) (lanes 3 and 6). Transfected cell extracts were immunoprecipitated with anti-Flag mAb for *in vitro* kinase assay using GST–CtIP(C) (lanes 1–3) and GST–CtIP(C-S664/745A) (lanes 4–6) as substrates. Top, western blot of ectopically expressed ATM

using anti-Flag mAb. Middle, Coomassie blue gel of GST–CtIP(C) and GST–CtIP(C-S664/745A). Bottom, autoradiogram of phosphorylated GST–CtIP(C) and CtIP(C-S664/745A). **c**, Phosphorylation of Ser 664 and Ser 745 of CtIP *in vivo* upon IR. U2OS cells transiently transfected with Flag-tagged CtIP(WT), CtIP(S664A) or CtIP(S664/745A) expression vectors, were metabolically labelled with ³²P, then either untreated or treated with IR (20 Gy). CtIP was isolated for tryptic phosphopeptide mapping analysis¹⁹. Two new spots appeared for CtIP(WT) upon IR (arrowheads). Mutation of Ser 664 to alanine resulted in the disappearance of one IR-induced spot, whereas mutation of both Ser 664 and Ser 745 to alanine resulted in the loss of both spots on the tryptic phosphopeptide map.

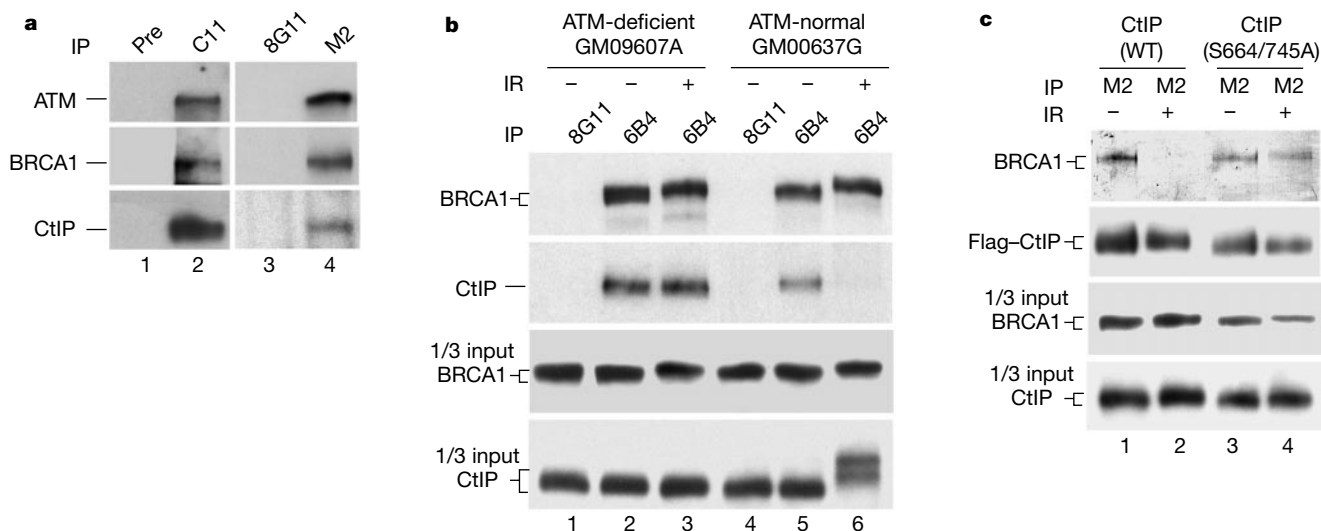


Figure 3 Phosphorylation of CtIP by ATM is essential for dissociation of CtIP from BRCA1 upon IR. **a**, Formation of ATM–BRCA1–CtIP complex *in vivo*. HCT116 cell lysates were immunoprecipitated using pre-immune serum (lane 1) or anti-CtIP polyclonal antibody, C11 (lane 2), and immunoblotted for ATM, BRCA1 or CtIP. In lanes 3 and 4, HCT116 cells were transiently transfected with Flag-tagged ATM expression construct. The lysates were immunoprecipitated using anti-GST (8G11) monoclonal antibody (mAb) as a control or anti-Flag mAb, and immunoblotted for ATM, BRCA1 or CtIP. **b**, Dissociation of BRCA1 and CtIP is abrogated in ATM-deficient cells upon IR. ATM deficient (GM09607A, lanes 1–3) or normal human fibroblasts (GM00637G, lanes 4–6) were untreated (–) or treated with

IR (+, 40 Gy). Extracts were immunoprecipitated using control (8G11) mAb or anti-BRCA1 (6B4) mAb, and immunoblotted for BRCA1 (top) or CtIP (bottom). Total input of BRCA1 and CtIP is also shown. **c**, CtIP phosphorylation mutant fails to dissociate from BRCA1 upon IR. Flag-tagged wild-type (lanes 1 and 2) or mutated (lanes 3 and 4) CtIP were transiently transfected into U2OS cells, and the cells were either untreated (–) or treated with IR (40 Gy). The extracts were immunoprecipitated using anti-Flag mAb (M2). Top, immunoblot for BRCA1; bottom, immunoblot for Flag-tagged CtIP. Total input of BRCA1 and Flag-tagged CtIP is also shown.

pitated with anti-Flag antibody (M2), and subjected to two-dimensional tryptic peptide analysis¹⁹. Two additional tryptic ³²P-phosphopeptide spots arose from CtIP(WT) immunoprecipitated from γ -irradiated cells compared with non-irradiated cells (Fig. 2c, top panel, arrows); however, both of these two ³²P-phosphopeptide spots were absent in CtIP(S664/745A), and only one of these ³²P-phosphopeptides spots was present in CtIP(S664A) (Fig. 2c, bottom panel). Together, these data indicate that both Ser 664 and Ser 745 on CtIP may be phosphorylated *in vivo* in response to IR.

To explore the biological significance of ATM-dependent phosphorylation of CtIP upon IR, we initially tested whether ATM associates with the CtIP and BRCA1 complex *in vivo* by co-immunoprecipitation. BRCA1, CtIP and ATM were co-immunoprecipitated from the human colon carcinoma cell line HCT116 using an anti-CtIP polyclonal antibody (Fig. 3a, compare lanes 1 and 2). As the ATM-specific antibody, 2C1, was not efficient for immunoprecipitation, HCT116 cells were first transfected with a Flag-tagged wild-type ATM expression vector, and subsequently immunoprecipitated with anti-Flag M2 antibody. BRCA1 and CtIP were both co-immunoprecipitated with Flag-tagged ATM (Fig. 3a, lane 4). These data suggest that ATM is present in the previously identified BRCA1–CtIP complex *in vivo*.

We then proceeded to evaluate the consequence of IR-induced

ATM-dependent phosphorylation of CtIP on CtIP–BRCA1 complex formation. In the absence of IR, CtIP associated with BRCA1 in both ATM-deficient (GM09607A) and ATM-normal (GM00637G) cells (Fig. 3b, lanes 2 and 5). Upon IR, CtIP dissociated from BRCA1 in ATM-normal cells, but not in ATM-deficient cells (Fig. 3b, compare lanes 3 and 6). Western blot analysis of the cell lysates clearly indicated that IR-induced hyperphosphorylation of CtIP is absent in ATM-deficient cells (Fig. 3b, '1/3 input' panel, compare lanes 3 and 6). Because ATM also phosphorylates BRCA1 (ref. 20), the persistent association of CtIP and BRCA1 in ATM-deficient cells after IR could be attributed to a deficiency in the phosphorylation of BRCA1, of CtIP, or of both. To discriminate between these alternatives, we performed similar experiments with U2OS cells transiently transfected with Flag-tagged CtIP(WT) or CtIP(S664/745A). BRCA1 associated with both CtIP(WT) or CtIP(S664/745A) in the absence of IR (Fig. 3c, lanes 1 and 3), whereas BRCA1 dissociated from CtIP(WT), but remained associated with CtIP(S664/745A) upon IR (Fig. 3c, compare lanes 2–4). Because U2OS cells contain wild-type ATM, IR-induced ATM-dependent phosphorylation of BRCA1 has no apparent effect on the status of the CtIP–BRCA1 complex (Fig. 3c). It should be noted that the change in electrophoretic mobility of Flag-tagged wild-type CtIP was less prominent after IR, perhaps due to the addition of the Flag epitope. These

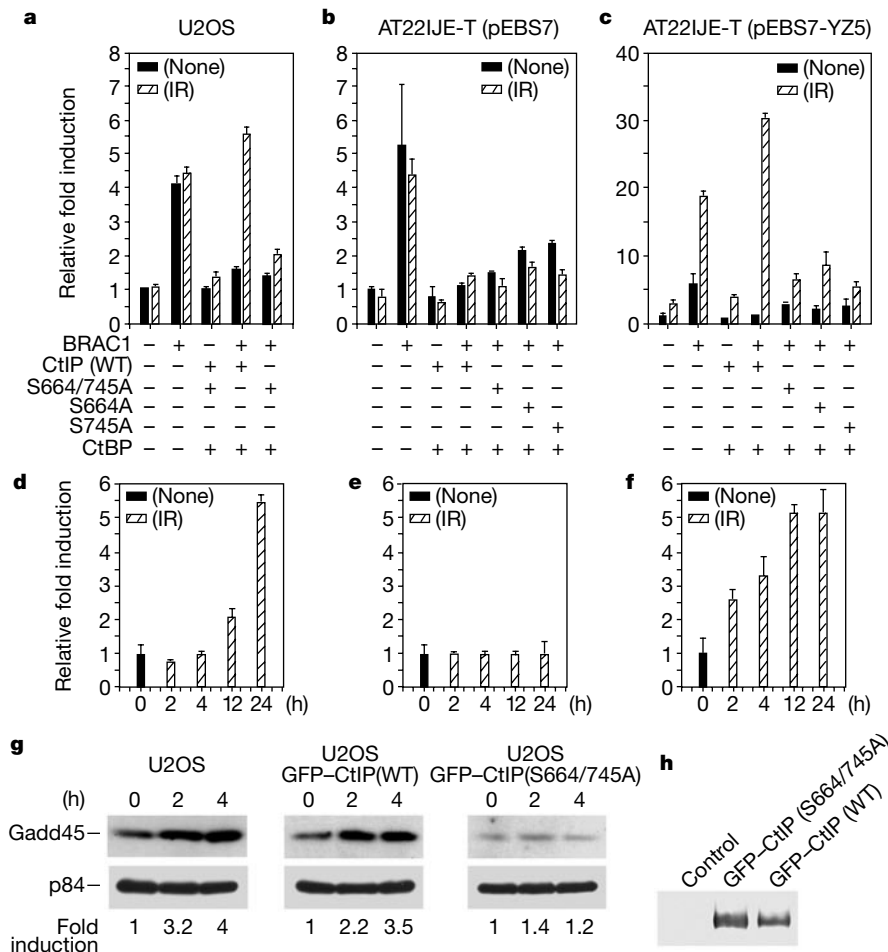


Figure 4 Regulation of GADD45 expression by BRCA1, CtIP and CtBP. **a–c**, U2OS, AT221JE-T (pEBS7) or AT221JE-T (pEBS7-YZ5) cells were co-transfected with pL3 (containing *GADD45* intron 3 driving expression of a luciferase reporter gene), pSV40- β -gal (control plasmid) and the plasmids indicated. The luciferase activity was measured and normalized with β -galactosidase activity. Scale in **c** is different from in **a** and **b**. Results were derived from three independent transfection experiments. **d–f**, pL3 reporter activity in U2OS, AT221JE-T (pEBS7), or AT221JE-T (pEBS7-YZ5) cells treated with IR. Cells were co-transfected with pL3 and pSV40- β -gal plasmid, and untreated (solid bar)

or treated (hatched bar) with IR (30 Gy) at 36 h after transfection. Transfected cells were then assayed for luciferase activity at the time points indicated. **g**, IR-induced expression of cellular GADD45 protein. U2OS cells stably expressing either GFP–CtIP (WT) or GFP–CtIP(S664/745A) were collected 2 and 4 h after IR and immunoblotted for GADD45 and a nuclear matrix protein p84 as an internal control⁹. Blots were developed using an ECL kit and quantified by an SI Densitometer (Molecular Dynamics). The protein ratio of GADD45:p84 is shown. **h**, Immunoblot analysis of ectopically expressed GFP–CtIP(WT) and GFP–CtIP(S664/745A) in the stable cell clones using anti-GFP antibody.

results recapitulate the behaviour of the CtIP–BRCA1 complex in γ -irradiated ATM-deficient cells (Fig. 3b), and suggest that ATM-dependent phosphorylation of CtIP on Ser664 and Ser745 is essential for its dissociation from BRCA1 upon IR.

Dissociation of the CtIP–CtBP co-repressor complex from BRCA1, leading to relief of transcription repression, could represent one of the mechanisms regulating the transcriptional activity of BRCA1 upon DNA damage. To test this hypothesis, we examined the effect of CtIP–CtBP on the transactivation activity of BRCA1 on the *GADD45* regulatory element (intron 3 of *GADD45*) which was induced by BRCA1 (ref. 8). Consistently, expression of BRCA1 activated transcription from the reporter plasmid (pI-3) containing intron 3 (+1,553 to +1,695) of *GADD45* by about fourfold compared with the pcDNA3.1 vector alone in U2OS cells (Fig. 4a). Co-expression of CtIP(WT) or CtIP(S664/745A) and CtBP with BRCA1 repressed this BRCA1-induced activity by about 70–80%. After IR, however, CtIP(WT) no longer repressed the activity of BRCA1, whereas CtIP(S664/745A) retained persistent repression (Fig. 4a). The same persistent repression was observed with both CtIP(WT) and CtIP(S664/745A) in ATM-deficient cells [AT221JE-T(pEBS7)] after IR (Fig. 4b). Re-introduction of wild-type ATM into these cells [AT221JE-T(pEBS7-YZ5)] eliminated repression mediated by the CtIP(WT)–CtBP complex after IR, whereas CtIP(S664/745A)–CtBP maintained its repression (Fig. 4c). These results suggest that a defect in the ATM-dependent phosphorylation of CtIP(S664/745A) inhibited the dissociation of CtIP(S664/745A) from BRCA1 upon DNA damage, leading to the continuous repression of transcriptional activity from the intron 3 of *GADD45*. Furthermore, it appears that phosphorylation of both Ser 664 and Ser 745 is required for dissociation, as a single mutant form of CtIP(S664A or S745A) maintained its repressive effect after IR treatment (Fig. 4b, c). It was noted that the overall fold induction of the reporter (pI-3) was higher in the AT221JE-T (pEBS7-YZ5) cells compared with in the U2OS cells in these experiments. This is because the reporter activity 4 h after IR was induced 2–3-fold in AT221JE-T (pEBS7-YZ5) cells but very little in U2OS cells (Fig. 4d, f). The transcriptional activity of the reporter construct alone in ATM-deficient cells [AT221JE-T(pEBS7)] remained constant after IR, which is consistent with previous results showing that induction of *GADD45* in response to IR requires ATM (compare Fig. 4e and f)²¹. Together, these data suggest that phosphorylation of CtIP by ATM is critical for releasing BRCA1 from its repressive state. Consistently, overexpression of BRCA1 could titrate the endogenous CtIP–CtBP repressor complexes and thereby liberate BRCA1 from repression (Fig. 4a–c).

The persistent repression exerted by CtIP(S664/745A) suggests

that it may act as a dominant-negative inhibitor of BRCA1-mediated transcriptional activity following IR. To test this possibility, we created U2OS cells stably expressing green fluorescent protein (GFP) tagged CtIP(WT) or CtIP(S664/745A), challenged these cells with IR, and assessed endogenous expression of *GADD45* by immunoblot analysis. Overexpression of CtIP(S664/745A) but not CtIP(WT) inhibited the induction of *GADD45* after IR (Fig. 4g). The expression of GFP-tagged CtIP proteins was confirmed by immunoblot analysis (Fig. 4h). These data further support the notion that phosphorylation of CtIP at Ser 664 and Ser 745 is important for BRCA1-mediated induction of *GADD45* in response to DNA damage.

In response to genomic insult, ATM probably transduces the DNA damage signal by phosphorylating downstream effector molecules involved in regulating cell-cycle progression or DNA damage repair. For example, IR-induced ATM-dependent phosphorylation of p53 and hMDM2 contributes to the activation of p53, leading to the induction of p21 and *GADD45* (refs 17, 18, 22). BRCA1 has also been demonstrated to induce expression of p21 and *GADD45* (refs 6–8). Our study suggests another DNA damage-response pathway in which the signal is transmitted through phosphorylation of CtIP by ATM, leading to dissociation of the CtIP–CtBP repressor complex from BRCA1, which in turn, activate transcription of *GADD45* (Fig. 5). This regulatory mechanism may also explain our previous results concerning repression by CtIP/CtBP on the expression of p21 mediated by BRCA1 (ref. 9). Apparently, BRCA1 and p53 are important in p21 and *GADD45* expression in response to IR. It is likely that both p53 and BRCA1 mediate synergistic and parallel pathways to ensure a proper cellular response to DNA damage.

These results provide a link between ATM and BRCA1 through CtIP, which may explain the increased risk for breast cancer in certain populations of ataxia telangiectasia heterozygotes^{23,24}. In the absence of functional ATM, the activity of BRCA1 may become dysregulated leading, in turn, to a defect in the cellular response to DNA damage, concomitant genomic instability and, ultimately, tumorigenesis. □

Methods

Plasmid constructs

The pCtIP–CtIP(WT) plasmid that expresses the Flag-tagged CtIP was generated by subcloning Flag-tagged CtIP cDNA into pcDNA3.1 vector (Invitrogen). The pCtIP–CtIP(S664/745A) with mutations at Ser 664 and Ser 745 was engineered by site-directed mutagenesis. pCMV–ATM expresses Flag-tagged wild-type ATM, whereas pCMV–ATM(D2870A) expresses mutant ATM with Asp 2,870 changed to alanine by site-directed mutagenesis. GST–CtIP(C) was constructed by inserting the carboxy-terminal fragment of CtIP (amino acids 324–897) into the *Sma*I site of pGEPK3 vector. GFP–CtIP contains the full-length CtIP cDNA downstream of GFP-tagged expression vector²⁵. pcDNA–BRCA1, pRcCMV–CtBP and pSV40– β -gal plasmids have been described⁹. The pI-3 plasmid was constructed as described⁸.

Cell treatment, immunoprecipitation and western blot analysis

Cells were incubated in fresh medium for 3 h and treated with different dosages of γ -ray (5–40 Gy), ultraviolet (1 mJ cm^{−2}) or 0.01% MMS. The cells were collected 1 h after treatment and lysed in Lysis 250 buffer. Immunoprecipitation and western blots were carried out as described⁹. We used the following antibodies: anti-CtIP mouse polyclonal antibody C11, anti-BRCA1 monoclonal antibody 6B4, anti-GST monoclonal antibody 8G11 (ref. 9), ATM monoclonal antibody 2C1 (GeneTex), anti-Flag monoclonal antibody M2 (Sigma), anti-*GADD45* rabbit polyclonal Ab H165 (Santa Cruz Biotechnology) and anti-GFP monoclonal antibody (Clontech).

λ -Phosphatase treatment

Immune complexes containing CtIP were washed in Lysis 250 buffer in the absence of phosphatase inhibitors. Parallel samples were resuspended in λ -phosphatase buffer (New England Biolabs) either in the presence or absence of phosphatase inhibitors, NaF (50 mM final concentration) and Na₃VO₄ (2 mM final concentration). λ -Phosphatase (400 U) was added to each sample followed by incubation at 30 °C for 1 h.

Transfection and luciferase assay

Plasmid DNA including 10 μ g of pCMV (vector), pCMV–ATM or pCMV–ATM(D2870A) was transfected into 293 cells (2×10^6 in 10-cm dish) by the calcium phosphate/DNA

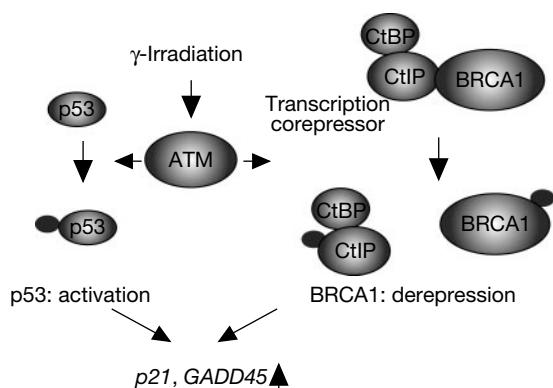


Figure 5 Model showing how ATM modulates the BRCA1 transcriptional regulation of DNA damage-response genes following IR. In response to IR, ATM kinase becomes activated and phosphorylates CtIP to disrupt the CtIP–CtBP–BRCA1 complex. Consequently, BRCA1 is released and participates in the activation of DNA damage-response genes *p21* and *GADD45*.

co-precipitation method. Transfection into HCT116 cells was performed using lipofectin. For measuring the transactivation activity by luciferase assay, U2OS cells were transfected by calcium phosphate/DNA co-precipitation with 0.5 µg of pI-3 reporter construct, along with 5 µg of pcDNA-BRCA1, 2.5 µg of pRcCMV-CtBP, 2.5 µg of pCNF-CtIP(WT), pCNF-CtIP(S664/745A), pCNF-CtIP(S664A), pCNF-CtIP(S745A), or pcDNA3.1 as the control vector to equalize the amount of the transfected DNA. One microgram of pSV40-β-gal was co-transfected for standardization of transfection efficiency by measurement of β-galactosidase activity. The cells were irradiated with 30 Gy 36 h after transfection, and assayed for luciferase and β-galactosidase activities 4 h after treatment using standard procedures⁹.

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